

MECHANISMS UNDERLYING TWO DIFFERENT FMRF
AMIDE INDUCED IONIC CURRENTS IN IDENTIFIED
NEURONES OF 'HELIX ASPERSA'

Stuart W. P. Falconer

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Mechanisms underlying two
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neurones of Helix Aspersa

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DEDICATION

I respectfully dedicate this research thesis to my wife Jacqueline and my family for their continued love and support during this project.

Abstract

Application of the molluscan neuropeptide FMRFamide to two identified neurones in the cerebral ganglia of Helix aspersa induces quite different effects. In the C1 neurone, FMRFamide produces a slow hyperpolarizing current carried by K^+ while with the C2 neurone it causes a fast depolarizing current carried by Na^+ . Possible mechanisms underlying the slow K^+ response were examined and the fast response was characterized using voltage clamp techniques. Some patch clamp experiments were also used for the slow K^+ response.

The slow K^+ response was shown to depend on a G protein, which was sensitive to inhibition by pertussis toxin, indicating that it was mediated by a G_i or G_o protein. Second messengers such as cyclic AMP, cyclic GMP, IP_3 , arachidonic acid and Ca^{2+} along with the activation of protein kinase C were all found not to be directly involved in producing the FMRFamide response. These negative results with the second messengers gave rise to the view that the FMRFamide receptors and K^+ ion channels may be linked directly through the activation of G proteins.

5-HT, probably acting through raised cyclic AMP levels, reduced the amplitude of the FMRFamide response which suggests that the channel opened by FMRFamide may be an "S" K^+ type channel. Activation of protein kinase C by phorbol ester also reduced the FMRFamide response. A role for protein phosphorylation was indicated by the use of okadaic acid which inhibits protein phosphatases 1 and 2A. Its application reduced the amplitude of the

FMRFamide response which suggested that increased protein phosphorylation levels lead to smaller responses. Thus, it seemed possible that protein phosphorylation levels controlled by cyclic AMP, protein kinase C activation and protein phosphatases 1 and 2A might modulate the activity of the receptor/ G protein/ ion channel complex. Alternatively, FMRFamide may operate through the activation of protein phosphatase(s) which reduce protein phosphorylation levels.

Patch clamp studies in cell attached mode on the C1 neurone failed to reveal any channel openings induced by FMRFamide. This result also tends to rule out the direct involvement of a second messenger.

The fast depolarizing FMRFamide response of the C2 neurone, which is due to the opening of a ligand gated channel, was found to be carried by Na^+ and not Ca^{2+} . Amiloride produced a reversible block of the current. Tetrodotoxin and lignocaine had no effect on the FMRFamide response while raised cyclic AMP levels potentiated the response. In the presence of okadaic acid and increased levels of cyclic AMP, the FMRFamide response is potentiated. This potentiation was not maintained in the presence of okadaic acid alone. The raised protein phosphorylation levels therefore did not cause potentiation, which suggested that cyclic AMP may have a direct effect on the receptor/channel complex.

Abbreviations

ACh	acetylcholine
ADP	adenosine diphosphate
AgCl	silver chloride
ATP	adenosine 5'-triphosphate
8-bromo cyclic GMP	8-bromo guanosine 3':5'-cyclic monophosphate
cyclic AMP	adenosine 3':5'-cyclic monophosphate
cyclic GMP	guanosine 3':5'-cyclic monophosphate
CCK	cholecystokinin
8-(-4) CPT cyclic AMP	8-(-4)chlorphenylthio adenosine-3':5'-monophosphate
DAG	1,2-diacylglycerol
dibutyryl cyclic AMP	N ⁶ ,2'-O-dibutyryl adenosine 3':5'-cyclic monophosphate
dibutyryl cyclic GMP	N ² ,2'-O-dibutyryl guanosine 3':5'-cyclic monophosphate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
DTT	dithiothreitol
EGTA	ethylene glycol bis-(aminoethylether) N,N,N',N'-teraacetic acid
epsps	excitatory postsynaptic potentials
GABA	gamma-aminobutyric acid
GDP	guanosine diphosphate
GMP	guanosine 5'-monophosphate
GTP	guanosine 5'-triphosphate
GTP γ S	guanosine 5'-O-(3-thiotriphosphate)
HEPES	N-2-hydroxyethylpiperazine-N'-2- ethanesulphonic acid
HETE	hydroxyeicosatetraenoic acid
12-HPETE	hydroperoxyeicosatetraenoic acid
HPLC	high performance liquid chromatography
5-HT	5-hydroxytryptamine
IBMX	3-isobutyl-1-methyl xanthine
ICa ²⁺	calcium current
ICl ⁻	chloride current
I _K	potassium current
IK _{Ca}	calcium dependent potassium current
IK _S	"S" potassium current

IK _v	voltage dependent potassium current
IP ₃	inositol 1,4,5-trisphosphate
LHRH	luteinizing hormone, releasing hormone
MET	mechano-electrical transduction
MOPS	(3-[N-Morpholine] propane sulfonic acid)
NAD ⁺	β-nicotinamide adenine dinucleotide
OCa ²⁺	zero calcium
ONa ⁺	zero sodium
PIP ₂	phosphatidylinositol 4,5 bisphosphate
PTX	pertussis toxin
RIA	radioimmunoassay
V _h	holding potential
V _m	membrane potential
V _p	patch potential

UNITS:

M	molar
mM	millimolar
μM	micromolar
μm	micrometer
v	volume
w/v	weight per volume
nl	nanolitres
pA	picoamp
nF	nanoFarads
kHz	kiloHertz
kΩ	kilo ohms
kP	kiloPascals
MΩ	mega ohms
V	volt
mV	millivolt
I	current
s	second
ms	millisecond
r	radius
cm	centimeter
r.p.m	revolutions per minute
w	weight
μg	microgram

°C degree centigrade

AMINO ACIDS:

Ala	Alanine
Arg	Arginine
Asp	Asparagine
Cys	Cysteine
Gln	Glutamine
pGlu	pyro Glutamate
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

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Chapter 1

Introduction

1.1 INTERNEURONAL COMMUNICATION

To perform its task of controlling and integrating the function of multicellular organisms, the nervous system must have rapid communication between its individual neurones. Interneuronal communication occurs at sites known as synapses.

Synapses are separated into two categories, electrical and chemical. Electrical synapses are those where currents produced by an impulse in the presynaptic nerve terminal pass directly to the postsynaptic neurone through a low resistance pathway. These electrical communication sites appear in electron micrographs as gap junctions. There is no measurable delay with this type of transmission. Chemical synapses operate through a mechanism whereby a chemical substance (neurotransmitter) is released from the presynaptic terminal and diffuses across the synaptic gap to the postsynaptic neurone. The neurotransmitter then binds to receptors on the postsynaptic neurone and alters the membrane conductance to specific ions. Transmission across chemical synaptic junctions involves time delays of fractions of milliseconds.

1.2 NEUROTRANSMITTERS

Neurotransmitters may be grouped into two broad categories. There are the low molecular weight compounds, examples of which are acetylcholine (ACh), noradrenaline (NA), dopamine, 5-hydroxytryptamine (5-HT), γ -aminobutyric acid (GABA), glutamate and glycine.

Neurones possess the necessary biochemical machinery to synthesize these compounds at the terminal release sites. Also, enzymes are present which allow the compounds to be quickly degraded and/or re-uptaken into the terminals following their release. Rapid turnover of these compounds is a great advantage since supplies of neurotransmitter can be rapidly replenished following vigorous or lengthy stimulation.

Peptides fall into the second category of neurotransmitter substances. For many years peptides have been established as chemical messengers in the endocrine systems of multicellular animals. They are released into the bloodstream or haemolymph and transported often considerable distances to their target sites (chemically addressed), where they can have long lasting actions. However, it is now generally accepted that following the isolation and identification of a great number of peptides from nervous tissue throughout the animal kingdom and examining their wide ranging actions, that they also fulfill the role of the neurotransmitter. They are commonly known as neuropeptides and will be referred to as such throughout this thesis.

Neuropeptides, unlike the low molecular weight neurotransmitters can not be synthesized at the presynaptic nerve terminals. Instead they are produced from larger precursors which are synthesized on ribosomes within the cell body. This means that the neuropeptides must be transported over a long distance down the axon to release sites at the synaptic

terminals. The amount of neuropeptide which can be transported is small compared with the amount of low molecular weight transmitter that can be synthesized or taken up at the terminal. Thus, if the available supply of neuropeptide at the terminals is not to be exhausted, only a small amount of neuropeptide can be released at any one time. It seems feasible that some of the roles of neuropeptides may have been superseded by low molecular weight transmitters where a rapid turnover of neurotransmitter is required.

1.3 NEUROMODULATORS

The terminology regarding the actions of neuropeptides is controversial. Due to the facts that these actions are often slow in timecourse, that the sites of action may be fairly widespread rather than localized and that coexistence with low molecular weight neurotransmitters is common, they are often referred to as neuromodulators rather than neurotransmitters. Since the term "neuromodulatory" has been used in numerous ways it is important to define what it means in the context of this thesis. A neuromodulator will refer to a neuropeptide which only influences the actions of other synaptic connections. The site of modulation may be postsynaptic where the neuropeptide can change the response of a cell to other neurotransmitters. Alternatively, it can be presynaptic where the neuropeptide alters the release of other

neurotransmitters from the terminals. Examples of neuromodulatory actions will be described later.

1.4 LOCALIZATION AND CHARACTERIZATION OF NEUROPEPTIDES

The localization and characterization of neuropeptides in nervous systems can be problematic, mainly because of the small amounts present in a given tissue. Nevertheless there are now numerous examples of neuropeptides present in nervous tissue.

One method frequently used to detect neuropeptides is that of immunohistochemistry. Although this approach is both useful and powerful, the results should be considered with caution because it is common for antisera to cross react with structurally related immunogens. Much of the early neuropeptide identifications were carried out using immunohistochemistry. However, with the development of highly sensitive radioimmunoassay (RIA) and high performance liquid chromatography (HPLC) techniques, it is now possible to purify and chemically identify neuropeptides from small numbers of neurones.

1.5 NEUROPEPTIDES OF VERTEBRATE NERVOUS SYSTEMS

A substantial amount of the present knowledge regarding the localization, characterization, distribution, release, receptors for, actions of, and roles for neuropeptides has been obtained from work

performed on vertebrate nervous systems. Therefore some of this information will be considered first of all.

1.5.1 LOCALIZATION, CHARACTERIZATION AND DISTRIBUTION

Since it is now known that there is a large number of neuropeptides present in vertebrate nervous systems, only a few examples are considered here.

In 1971 the chemical structure of substance P, a member of the tachykinin neuropeptide family was first determined (Chang et al, 1971). Fairly soon after this, Otsuka et al (1972) isolated a peptide from the dorsal root of bovine spinal cord. This was identified as substance P. Considerable amounts of substance P have been identified using immunohistochemical techniques in a certain population of primary sensory neurones of the cat, namely the C group which are thin nerve fibres. These nerve fibres are associated with pain transmission, which points to at least one physiological function of substance P (Hokfelt et al, 1975). Ten years after the discovery of substance P, it was still the only tachykinin isolated from mammalian sources. In 1983, two novel tachykinin peptides, neurokinin A and neurokinin B were discovered (Kimura et al, 1983).

Since this discovery of neurokinin A and neurokinin B in the rat, studies of the regional distribution of these neuropeptides have been carried out and compared with substance P (Kanazawa et al, 1984; Arai and Emson, 1986). Substantial amounts have

been found in the substantia nigra, striatum, spinal cord, cerebral cortex and cerebellum. Although the amount of neurokinin A present in the tissues was relatively small compared to substance P, both neuropeptides exhibited nearly the same distribution pattern. Neurokinin B was found in considerably higher amounts than the other neuropeptides in the cerebral cortex.

Another neuropeptide found at its highest level in the cerebral cortex of the rat was cholecystokinin (CCK). In the regional distribution study performed by Beinfeld et al (1981), intermediate levels were also found in the striatum, thalamus and hypothalamus. Most of the work performed on CCK has concentrated on the octapeptide known as CCK-8S, although other forms do exist. CCK-8S appears to be the most abundant form found in brain extracts (Larsson and Rehfeld, 1979). CCK is present in the nervous system at concentrations that exceed the other neuropeptides (Iversen, 1983).

A few years after the discovery of substance P, endogenous opiate neuropeptides known as Met-enkephalin and Leu-enkephalin were also isolated from mammalian brain (Hughes et al, 1975).

When considering the localization, characterization and distribution of neuropeptides an important point to emphasize is that in most circumstances, they are co-localized with lower molecular weight neurotransmitters. A good example of co-localization is provided in the bullfrog sympathetic ganglion. Neurones in some ganglia of the lumbar chain

contain ACh and a luteinizing hormone releasing hormone (LHRH)-like peptide (Branton et al, 1986). The significance of this co-localization will be described in a later section of this chapter.

1.5.2 NEUROPEPTIDE RELEASE

It is important to know if neuropeptides are released from presynaptic terminals in a Ca^{2+} dependent manner following depolarization, as is the case with low molecular weight neurotransmitters. Evidence has shown that this is indeed what happens with several neuropeptides.

Henderson et al (1978) examined the release of Met- and Leu- enkephalin from synaptosomes produced from the corpus striatum. They found using the mouse vas deferens as a bioassay system that the basal release of enkephalin was greatly increased during depolarizations induced by high K^+ concentrations, or veritridine. When the Ca^{2+} concentration was reduced then the amount of enkephalin released was much smaller.

In perfused preparations of rat frontal lobe and striatum, neurokinin A and neurokinin B were shown to be released under high K^+ concentrations in the presence of Ca^{2+} , as was substance P (Linderfors et al, 1985). CCK was released in a Ca^{2+} dependent manner from rat synaptosomes and brain slices in the presence of high K^+ concentrations (Dodd et al, 1980).

1.5.3 NEUROPEPTIDE RECEPTORS

The existence of multiple receptors for the binding of released neuropeptides in the nervous system are now well documented, two examples of which are given below.

Receptors for the three mammalian tachykinins have now been divided into three types, namely NK-1 (substance P-suceptive), NK-2 (neurokinin A-suceptive) and NK-3 (neurokinin B-suceptive). These receptors have been located in the areas close to where the endogenous tachykinins are distributed in the nervous system. Interestingly, Masu et al (1987) have been able to express the gene encoding the receptor of neurokinin A in Xenopus oocytes and to deduce the amino acid sequence of the receptor protein using the combination of electrophysiological and cloning techniques. The protein structure anticipated from the primary structure is a seven-transmembrane segment feature and belongs to the superfamily of the GTP-binding protein group (G protein). Also Segawa and Nakata (1991) have shown that substance P receptors may couple with pertussis toxin sensitive G proteins. This implies that these neuropeptides operate through one, or more, of the well characterized signal transduction mechanisms.

Multiple receptors have also been identified for the endogenous opioid neuropeptides. These are known as μ , δ and κ (Martin et al, 1976; Lord et al, 1977). It was shown by Snyder (1980) that binding sites for opioid neuropeptides are close to neuronal terminals

which are immunoreactive for enkephalin and to involve areas of the central nervous system which have been linked with opioid actions. Association of μ and κ receptor types with signal transducing G proteins has been shown (Ueda et al, 1991). As with substance P, this suggests that the opioid neuropeptides may operate through one or more of the well known signal transduction mechanisms. The situation with the δ opioid receptor is different in that it does not pass on a signal to the G proteins, despite some interaction between them. Therefore, this is not a functional association between G protein and receptor as regards signal transduction.

1.5.4 EFFECTS OF NEUROPEPTIDES ON VERTEBRATE NEURONES

Many studies have been performed to determine how neuropeptides influence vertebrate neuronal activity as neurotransmitters and to elucidate when possible, the intracellular mechanisms underlying these effects. With the availability of intracellular recording and patch clamp techniques, substantial quantities of endogenous neuropeptides, selective receptor agonists and antagonists and various powerful intracellular biochemical probes, a vast amount of important data has now been published. A selection of these data will be considered in this section.

A proposed physiological role for substance P is that of a pain transmitter (Hokfelt et al, 1975). This neuropeptide has also been established as an excitatory

transmitter. When spinal cord preparations of new-born rats were subjected to substance P application, a depolarizing effect was observed (Konishi and Otsuka, 1974). With bullfrog lumbar sympathetic neurones under current clamp conditions, substance P produced a depolarization which was associated with an increase in membrane input resistance. When these neurones were voltage clamped the neuropeptide induced an inward current which was due to the inhibition of a voltage-dependent K^+ current, otherwise known as the M-current (Adams *et al*, 1983). The excitatory action of substance P observed on cultured magnocellular cholinergic neurones from newborn rat brains was found to be due to the reduction of an inwardly rectifying K^+ conductance (Stanfield *et al*, 1985). The steps between the binding of substance P to its receptor and the closure of the K^+ channels were not investigated, although it was suggested that an intracellular second messenger system is probably involved. Very little is known about the intracellular events which underlie the effects of the tachykinin neuropeptides on neurones. However, there is evidence which indicates that these neuropeptides stimulate the inositol phospholipid system in rat salivary glands (Hanley *et al*, 1980).

Neurokinin A and neurokinin B, similar to substance P, have been shown to have depolarizing effects on isolated spinal cord neurones of the new born rat (Matsuto *et al*, 1984).

When the endogenous opioid neuropeptides Met-enkephalin and β -endorphin were applied to neurones of

the rat locus coeruleus and the guinea pig ileum submucous plexus, they induced a concentration dependent hyperpolarization. This registered as an outward current when the neurones were voltage clamped close to their resting potential (North et al, 1987). It was due to the increased conductance of an inwardly rectifying K^+ current. Specific agonists of μ and δ receptors were used on neurones of both tissues and the conclusion reached was that the rat locus coeruleus neurones express only μ receptors and that the guinea pig submucous neurones express only δ receptors. It was shown with the non-hydrolysable analogue of GTP, GTP γ S, that a G protein was involved in the coupling between both opioid receptors and the K^+ channel. There was no evidence to suggest that activation of cyclic AMP dependent protein kinase, or protein kinase C, were involved in the increase in K^+ conductance. The involvement of other second messenger systems was not tested.

Since then, the μ -opioid receptor has been shown to activate inwardly rectifying K^+ conductances through a pertussis toxin sensitive G protein in rat locus coeruleus neurones (Williams et al, 1988). Of the two types of G protein which are sensitive to pertussis toxin, namely G_i and G_o , G_i was negatively coupled to the adenylate cyclase enzyme. The μ -opioid receptor has also been shown to inhibit adenylate cyclase in human neuroblastoma SH-SY5Y cells (Kazmi and Mishra, 1987). Activation of μ -opioid receptors in SH-SY5Y cells inhibited an N-type Ca^{2+} channel (Seward et al, 1991).

This group found that although coupling between the μ -receptor and Ca^{2+} channel involved a pertussis toxin sensitive G protein, inhibition of adenylate cyclase did not occur.

The action of a LHRH-like neuropeptide on bullfrog sympathetic neurones has provided an interesting insight into the concept of co-localization of neuropeptides and low molecular weight neurotransmitters. It is known that neurones in some lumbar chain ganglia contain the LHRH-like neuropeptide along with ACh and it appears that preganglionic C-fibres release both substances (Branton et al, 1986). However, while ACh acts only on the C-cells which are synaptically connected to preganglionic C-fibres which release ACh, the LHRH-like neuropeptide acts not only on some C-cells but also B-cells which are not in synaptic contact with the C-fibres. The electrophysiology has shown that ACh causes fast excitatory postsynaptic potentials (epsps) and the LHRH-like neuropeptide induces non cholinergic, late slow epsps. Therefore, although both ACh and the LHRH-like neuropeptide are released from the same neurones, they can operate on different target cells and cause different electrophysiological effects on these neurones. The individual effects of these neurotransmitters are clearly dependent upon the differential distribution of receptors on target cells and the ability of the LHRH-like neuropeptide to survive substantial diffusional distances (up to $10\mu\text{m}$ when operating on B-cells).

Somatostatin is another neuropeptide which has effects on neuronal M currents. However, whereas substance P inhibited the M current in bullfrog sympathetic neurones, somatostatin augmented the M current in rat hippocampal neurones (Schweitzer *et al*, 1990). This group showed that the second messenger pathway involving arachidonic acid underlies the effects of somatostatin in these neurones. In this pathway, arachidonic acid is produced by the action of phospholipase A₂ on esterified stores of membrane phospholipids. Arachidonic acid may then be further metabolized to leukotrienes by lipoxygenase enzymes. Oxidized arachidonic acid metabolites such as the leukotrienes are known as eicosanoids. It was shown that arachidonic acid and leukotriene C₄ mimicked the effects of somatostatin. Also, inhibitors of phospholipase A₂ abolished the effects of somatostatin. This was thought to be the first electrophysiological evidence for eicosanoid mediation of a neurotransmitter effect in a vertebrate central neurone.

The effect of CCK-8S on a subtype of neurone located in the ventromedial nucleus of the hypothalamus was investigated by Boden and Hill (1988). This brain region was examined since binding sites for CCK-8S are known to be present (Day *et al*, 1986) and CCK is believed to play a role in appetite regulation in this instance (Baile *et al*, 1986). CCK-8S induced excitation of these neurones which were already spontaneously active through the activation of CCK receptors. When the neurones were voltage clamped, it was shown that

this excitation is due in part to the reduction of an M current via a pertussis toxin insensitive G protein.

A good example where the intracellular mechanism underlying the excitatory effect of a neuropeptide on vertebrate neurones has been examined, is that of bradykinin on cultured rat dorsal root ganglion (DRG) neurones (Burgess et al, 1989). Bradykinin is a pain producing peptide formed at the site of injury and stimulates peripheral terminals of sensory afferent fibres. When it was applied to the DRG neurones, there was a depolarizing inward current with an increase in membrane conductance and it was thought that this was probably due to the opening of Na^+ channels. An additional effect of bradykinin was the uptake of $^{45}\text{Ca}^{2+}$ into the neurones through Ca^{2+} channels. When protein kinase C was activated by phorbol esters, there was a mimic of both the depolarization and $^{45}\text{Ca}^{2+}$ uptake of the neurones. When protein kinase C was either blocked with staurosporine or down regulated with prolonged exposure to phorbol esters, the effects of bradykinin were no longer seen. In addition, bradykinin also activated phospholipase C in DRG neurones which elevated inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) levels. The latter compound is an endogenous activator of protein kinase C, thus it is thought that protein kinase C activation may underlie some effects of bradykinin in sensory neurones.

1.5.5 MODULATORY ROLES OF NEUROPEPTIDES ON VERTEBRATE NEURONES

Neuropeptide Y has been shown to have important modulatory effects as regards the release of neurotransmitter and alteration of Ca^{2+} currents in different neurones. Using cultured DRG neurones Walker et al, (1988) found that neuropeptide Y potently inhibited Ca^{2+} currents (transient and sustained) under voltage clamp conditions. Pre-treatment of the neurones with pertussis toxin blocked the effects of neuropeptide Y hence implicating the involvement of a G protein (G_i or G_o). When Ca^{2+} influx was determined using microspectrofluorimetry it was found that depolarization induced influxes were partially inhibited by neuropeptide Y. Consistent with the results on Ca^{2+} currents, neuropeptide Y also inhibited the depolarization induced release of substance P from DRG neurones. It was suggested that this neuropeptide may be an important regulator of sensory function in vitro.

Members of the same group (Ewald et al, 1988) investigated the intracellular events which might underlie Ca^{2+} current inhibition in these neurones by neuropeptide Y. They found that protein kinase C was important, although the exact role of this enzyme may depend on the type of Ca^{2+} channel involved.

The actions of neuropeptide Y have also been examined in dissociated cultured vagal sensory (nodose) neurones (Wiley et al, 1990). These neurones have three

Ca^{2+} current components; a transient low threshold (T) current, a slowly inactivating high threshold (L) current and a transient high threshold (N) current. Neuropeptide Y reduced the N current component and inhibited K^+ evoked release of ACh. When the cultured neurones were pre-treated with pertussis toxin, these effects of neuropeptide Y were blocked. The results indicated that the entry of Ca^{2+} through N-type channels into these neurones may be coupled selectively to the release of ACh, and that neuropeptide Y can inhibit the release by acting through a pertussis toxin sensitive G protein.

Selective agonists for opioid receptors (μ , δ or κ) modulate the electrically stimulated release of Met-enkephalin from guinea pig myenteric plexus. Low doses of opioids enhance release whereas higher concentrations inhibit release. The mechanism of action of this modulation has been investigated by Gintzler and Xu, (1991). Whilst pre-treatment of the preparation with pertussis toxin reduced the potency of μ , δ , or κ receptors to selective agonists to inhibit the release of enkephalin, it did not effect the enhancement of release. In contrast, when the preparation was pretreated with cholera toxin, inhibition of release was unaffected but enhancement of release was abolished. These results showed that a pertussis toxin sensitive G protein (G_o or G_i) mediates the inhibition of release by opioids, and a cholera toxin sensitive G protein (G_s) mediates enhancement of release.

Somatostatin is a neuropeptide which has been shown to have a modulatory action on neurones of the rat hippocampus and parietal cortex that can respond to ACh (Mancillas et al, 1986). Both of these brain regions contain areas where somatostatin and cholinergic nerve terminals overlap. It was discovered that somatostatin application alone inhibited spontaneous firing of nearly all neurones tested whereas, ACh alone enhanced their firing. When both substances were applied simultaneously, the ACh excitations were enhanced by 78%. The ability of somatostatin to produce this effect was dose-dependent and also seemed to be specific for ACh, since it did not enhance the responsiveness to pulses of the excitatory amino acid glutamate. The site of modulation, whether pre- or postsynaptic, was not determined.

The action of neurotensin on autoreceptor-mediated dopamine effects on rat midbrain dopamine cell activity (Shi and Bunney, 1991) is another interesting example of a neuropeptide modulatory action. In this case, the concept of autoreceptors is used which means a neurone having receptors for, and its activity being regulated by a transmitter substance released by itself. Neurotensin produced a marked attenuation of the inhibitory effect of either dopamine or the specific D₂ agonist quinpirole. The intracellular events underlying this modulatory effect remain to be elucidated.

1.6 NEUROPEPTIDES OF INVERTEBRATE NERVOUS SYSTEMS

1.6.1 ISOLATION AND CHARACTERIZATION

The major problem facing investigators interested in isolating and characterizing neuropeptides from invertebrate animals has been the small quantities present in their nervous systems. In early studies it was a regular occurrence to use hundreds of animals to purify tiny amounts of neuropeptide. Given these difficulties it was surprising to find that one of the first peptides sequenced was eleudoisin which originated from salivary glands of the octapod Eledone (Erspamer and Anastasi, 1962). However, it should be noted that salivary glands provided a larger bulk of tissue compared to neuronal tissue. Eleudoisin was the first member of the tachykinin family to be isolated. The tachykinin family of neuropeptides also include substance P, neurokinin A and neurokinin B.

A number of endogenous invertebrate neuropeptides have now been isolated and characterized. Many of them originate from arthropod and molluscan species. Proctolin and the Phe-Met-Arg-Phe-NH₂ (FMRFamide) related peptides have probably been studied in the most detail. A great deal of information has been presented, not only regarding their localization and characterization, but also their physiological effects on nerve and muscle tissue. Since the experiments described in this thesis are concerned with the actions

of FMRFamide on identified neurones, attention will now be focussed on the FMRFamide-related peptides.

1.6.2 DISCOVERY OF FMRFamide

When extracts of molluscan ganglia were studied by Frontali, Williams and Welsh (1967), most of the cardioactive material was found in the chromatographic fraction which was designated as "peak c". They also discovered that these partially purified substances were susceptible to proteolytic enzyme breakdown, hence indicating their peptidergic nature. Price and Greenberg, (1977) isolated peak c from ganglia extracts of the sunray clam Macrocallista nimbosa. This was achieved using the the heart of the clam Mercenaria mercenaria and the radula protractor muscle of Busycon as bioassays. Analysis of peak c material was carried out using solvent extractions, gel filtration and ion exchange chromatography. It was found to consist of the amidated tetrapeptide Phe-Met-Arg-Phe-NH₂ following amino acid analysis. This neuropeptide is referred to as "FMRFamide" using the single letter abbreviation system for the amino acids comprising its structure.

As people became more interested in FMRFamide, it became clear that it was only one member of an expanding family of related neuropeptides. Most of the FMRFamide-related peptides are extended at the N-terminus and have a leucine (Leu, L) substitution for methionine (Met, M) at position 2 of the FMRFamide

molecule. The tetrapeptide FLRFamide is also a member of this family.

1.6.3 LOCALIZATION, ISOLATION AND CHARACTERIZATION OF THE FMRFamide RELATED PEPTIDES

Evidence has shown that the FMRFamide related peptides are present in many different species. Antibodies raised against the C-terminus of the FMRFamide molecule for histochemical studies have revealed immunoreactive material in nerve and muscle tissue of molluscs (Cardot and Fellman, 1983; Schot *et al*, 1984; Elekes and Nassel, 1990; Fujiwara *et al*, 1991), insects (Carroll *et al*, 1986; Wather and Schafer, 1988; Robb and Evans, 1990; Sivasubramanian, 1991), coelenterates (Grimmelikhuijzen and Spencer, 1984; Carlberg *et al*, 1989; Koizani *et al*, 1989), crustaceans (Calloway *et al*, 1987; Mercier *et al*, 1991), flatworms (Reuter *et al*, 1984), nematodes (Davenport *et al*, 1988) and vertebrates (Sasek and Elde, 1985; Muske *et al*, 1987; Ostholm *et al*, 1990; Kivipelto and Panula, 1990; Chiba *et al*, 1991). The more direct approach of isolation and characterization has revealed a variety of neuropeptides which are likely to be FMRFamide related. These have been found in a number of different animals, some of which are listed along with the neuropeptides in Table 1.1.

Table 1.1

A list of FMRFamide related peptides which have been isolated and characterized from a variety of animals.

Arthropods

- (a) locust :
Pro-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe-NH₂
(schisto FLRFamide)
- (b) cockroach :
pGlu-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe-NH₂
(leucomyosuppressin)
- (c) lobster :
Thr-Asn-Arg-Asn-Phe-Leu-Arg-Phe-NH₂ (F₁)
Ser-Asp-Arg-Asn-Phe-Leu-Arg-Phe-NH₂ (F₂)
- (d) drosophila :
Asp-Pro-Lys-Gln-Asp-Phe-Met-Arg-Phe-NH₂

Coelenterates

- (e) jellyfish :
Glu-Leu-Leu-Gly-Gly-Arg-Phe-NH₂
(Pol-RFamide)
- (f) sea anemone :
pGlu-Gly-Gly-Arg-Phe-NH₂ (Antho-RFamide)

Vertebrates

- (g) chicken :
Leu-Pro-Leu-Arg-Phe-NH₂
- (h) oxen :
Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH₂

Ala-Gly-Glu-Gly-Leu-Ser-Ser-Pro-Phe-Trp-Ser-Leu-
Ala-Ala-Pro-Gln-Arg-Phe-NH₂

References

- (a) Robb et al, 1989; (b) Holman et al, 1986;
(c) Kravitz et al, 1987 (F₁); Trimmer et al, 1987 (F₂);
(d) Nambu et al, 1988; (e) Grimmelikhuijzen et al,
1988; (f) Grimmelikhuijzen and Graff, 1986; (g) Dockray
et al, 1983; (h) Yang et al, 1985.

1.6.4 FMRFamide RELATED PEPTIDES OF HELIX

In 1981 Cottrell, Price and Greenberg analysed whole ganglia extracts from Helix aspersa for FMRFamide-like activity. They used chromatographic methods along with bioassays which were Busycon radula protractor muscle, Mercenaria heart and Helix heart. When pooled ganglia extracts were chromatographed with FMRFamide, the biological activities did not elute from the column at the same time. Also, the relative potency of the purified peptide compared to FMRFamide on the Busycon muscle and Mercenaria heart was about the same, but on the Helix heart was 600 times more potent. Although FMRFamide-like activity originating from the ganglia had clearly been shown, this activity did not appear to be due to the action of authentic FMRFamide. Purified extracts of the identified F1 neurone were also shown to have FMRFamide-like activity.

Price et al, (1985) screened extracts of Helix ganglia for FMRFamide related peptides. Several peaks of FMRFamide-like immunoreactivity eluted from a HPLC column. One of these peaks co-eluted with oxidized FMRFamide and another with FMRFamide itself. This was evidence that authentic FMRFamide is present in Helix ganglia. The peptide purified and sequenced in this study was pGlu-Asp-Pro-Phe-Leu-Arg-Phe-NH₂ (pQDPFLRFamide), a FMRFamide related heptapeptide. This heptapeptide was 100 times more potent than FMRFamide on isolated Helix heart but slightly less potent on the Busycon radula protractor muscle.

Lehman and Price, (1987) looked at the localization of FMRFamide related peptides in different tissues of Helix aspersa. They found FMRFamide-like activity concentrated in the nervous system, male reproductive tract, tentacles and posterior digestive system. Immunohistochemical investigations confirmed these localizations. When the tissue extracts were analysed by gel chromatography, HPLC and bioassay, they were found to contain both FMRFamide and the extended heptapeptide pQDPFLRFamide.

More recently, Price et al (1990) have identified seven FMRFamide related peptides in both whole Helix aspersa and isolated ganglia. A list of these neuropeptides is given Table 1.2 showing their amino acid sequences and single letter abbreviations. When bioassayed on Helix heart the heptapeptides were more potent than the tetrapeptides.

A system thought to be ideal for studying FMRFamide as a neurotransmitter substance was investigated by Cottrell et al (1983). An identified neurone in each cerebral ganglion of Helix aspersa, the C3, stained positively with antisera for FMRFamide. When the C3 neurones were subjected to RIA, the results confirmed the presence of FMRFamide-like activity. The processes of the C3 neurones were traced in the ganglia and associated nerve trunks. Several branches passed along to the tentacle retractor muscle. Stimulation of the C3 neurone evoked a smooth contraction of the muscle while exogenously applied FMRFamide produced rhythmic rather than smooth muscle contractions.

Table 1.2

The FMRFamide related peptides of Helix aspersa.
The single letter abbreviations for the amino acid
sequences of the neuropeptides are shown.

Phe-Met-Arg-Phe-NH₂

F M R F amide

Phe-Leu-Arg-Phe-NH₂

F L R F amide

pGlu-Asp-Pro-Phe-Leu-Arg-Phe-NH₂

pQ D P F L R F amide

Asn-Asp-Pro-Phe-Leu-Arg-Phe-NH₂

N D P F L R F amide

Ser-Asp-Pro-Phe-Leu-Arg-Phe-NH₂

S D P F L R F amide

Asn-Asp-Pro-Tyr-Leu-Arg-Phe-NH₂

N D P Y L R F amide

Ser-Glu-Pro-Tyr-Leu-Arg-Phe-NH₂

S E P Y L R F amide

Exogenously applied ACh, although 100 times less potent than FMRFamide, evoked a smooth contraction.

Xu et al, (1989) demonstrated that the C3 neurone contained ACh along with the target muscle and could produce ACh from its usual precursors. Bewick et al, (1990) found that FMRFamide was the only FMRFamide related peptide present in the C3 neurone. Using intracellular recording methods ACh was shown to evoke depolarization of the muscle fibres whilst FMRFamide had no effect on membrane potential. Therefore, although FMRFamide has been positively identified in the C3 neurone, its role in this system remains to be elucidated.

1.6.5 FMRFamide RELATED PEPTIDES OF LYMNEA AND APLYSIA

Members of the FMRFamide related family of neuropeptides in Helix are by no means confined to this molluscan species. For example, in ganglia of the snail Lymnea stagnalis FMRFamide, SDPFLRFamide and GDPFLRFamide (see Table 1.2 for amino acid sequences) have been identified (Ebberink et al, 1987). FMRFamide has been purified and sequenced in ganglia of Aplysia (Lehman et al, 1984) and the precursor protein for FMRFamide has been found in Aplysia (Scheller and Kirk, 1987). It contains 28 copies of FMRFamide and 1 copy of FLRFamide.

Indirect evidence was presented by Schacher et al, (1985) that FMRFamide might be released from an Aplysia neurone which is known to be cholinergic. The giant

cholinergic R2 neurone was cultured with the L11 and R15 neurones from the abdominal ganglion. The L11 and R15 neurones receive a cholinergic input from other neurones in vivo, but not from the R2. When in culture, the R2 reliably formed unidirectional chemical connections with these neurones. The connection between the R2 and L11 neurones had both a fast inhibitory and a slow excitatory component. The former effect was shown to be cholinergic but the latter was not. This indicated that the slow excitatory response was evoked by a non-cholinergic transmitter. A similar situation arose with the R2-R15 connection. Action potentials in the R2 neurone produced a slow inhibitory response in the R15 neurone, an effect which was not cholinergic. The fast excitatory response normally observed for cholinergic input onto the R15 neurone in the intact ganglion was not seen. The R15 in culture did have cholinergic receptors since ACh application evoked a fast excitatory response of this neurone. Thus the R2 must also have been releasing a non-cholinergic transmitter onto the R15 neurone.

Previous reports had shown that the R2 neurone contained immunoreactivity for FMRFamide (Brown et al, 1984) and that this neuropeptide was able to produce both inhibitory and excitatory actions on abdominal ganglion neurones (Stone and Mayeri, 1981). It seemed that FMRFamide was therefore a strong candidate for the non-cholinergic transmitter released by the R2 neurone.

1.6.6 EXPRESSION OF FMRFAMIDE RELATED PEPTIDES IN LYMNEA AND HELIX

In Lymnea it has been shown by Linacre et al, (1990) that a single gene encodes for a precursor protein which contains 9 copies of FMRFamide, 2 copies of FLRFamide and 2 putative pentapeptides, Glu-Phe-Leu-Arg-Ile-NH₂ (EFLRIamide) and pGlu-Phe-Tyr-Arg-Ile-NH₂ (pQFYRIamide). Saunders et al, (1991) found that an exon 3' to the FMRFamide/FLRFamide encoding region encoded 7 copies of GDPFLRFamide and 6 copies of SDPFLRFamide. Another 3 peptides encoded by this exon were Glu-Phe-Phe-Pro-Leu-NH₂ (EFFPLamide), SDPYFLRFamide and Ser-Asp-Pro-Phe-Phe-Arg-Phe-NH₂ (SDPFFRFamide). The conclusion reached was that the region encoding the extended peptides makes up one exon of an extended gene. Preliminary analysis also showed that GDP-/SDP-FLRFamide mRNA was expressed in discrete neurones in the CNS. It is possible that some neurones may express only extended peptides and others only tetrapeptides.

Two copy DNA (cDNA) clones encoding the FMRFamide related peptides have been isolated from Helix aspersa ganglia (Lutz et al, 1991). They were named HF1 and HF4. On the HF1 message there are 10 copies of FMRFamide and 1 copy of FLRFamide. The HF4 cDNA clone encodes the extended peptides and some novel peptides. It remains to be determined whether the regions encoding the tetrapeptides and the extended peptides are located on 1 gene or 2 separate genes, but it is

clear that there are different species of mRNA and that each is differentially expressed in different neurones.

1.6.7 FMRFamide RELATED PEPTIDES OF HELISOMA

The CNS of the pond snail Helisoma has been shown to contain FMRFamide, FLRFamide and GDPFLRFamide (Bulloch et al, 1988).

Richmond et al, (1991) have found that the peptidergic synapse formed between the identified central neurones of Helisoma, VD4 and its target P1, has proved to be an ideal system with which to examine the release and postsynaptic effects of FMRFamide related peptides. The VD4 neurone was shown to contain FMRFamide along with smaller amounts of FLRFamide and GDPFLRFamide. The action of these neuropeptides were examined on the postsynaptic P1 neurone in culture conditions. FMRFamide and FLRFamide reduced the macroscopic high-voltage-activated (HVA) Ca^{2+} current while GDPFLRFamide had only small effects at higher concentrations. The reduction of the Ca^{2+} current by FMRFamide was found to be irreversible in the presence of GTP γ S, a non-hydrolysable analogue of GTP. This indicated that these FMRFamide related peptides acted through receptor coupled G proteins to reduce the HVA Ca^{2+} current.

The synaptic connection between VD4 and P1 was simulated in culture. Fura-2 was used to monitor the action potential evoked Ca^{2+} transient in neurites growing from P1 neurones. Applications of FMRFamide,

FLRFamide and GDPFLRFamide all reduced the magnitude of this transient. This action of the neuropeptides was mimicked by stimulation of the VD4 presynaptic neurone. There were also localized reductions in the action potential evoked Ca^{2+} transients in the P1 neurone at the points of contact between the neurites of VD4 and P1. These results indicated that FMRFamide related peptides released from VD4 neurones reduced the Ca^{2+} influx of neurone P1.

Stimulation of VD4, as well as reducing Ca^{2+} influx in the P1 neurone also reduced the excitability of the P1. Initial experiments by Richmond (unpublished) indicated that this was due to an increased K^{+} conductance. Although this effect remains to be characterized, it seems likely that the FMRFamide related peptides from the VD4 neurone hyperpolarize the P1 neurone by increasing a K^{+} conductance.

1.6.8 FMRFamide RELATED PEPTIDES IN VERTEBRATES

The FMRFamide related peptides which have been isolated and sequenced from vertebrate animals are listed in Table 1.1. The pentapeptide Leu-Pro-Leu-Arg-Phe- NH_2 (LPLRFamide) which was identified in chicken brain (Dockray et al, 1983) proved to be the first vertebrate peptide to have been discovered by its interaction with an antiserum to an invertebrate neuropeptide. Biological activity of this neuropeptide was examined on two systems and compared to FMRFamide. Intravenous injection of the pentapeptide into

anaesthetized rats produced a rapid increase in arterial blood pressure which had a similar time course to that of FMRFamide. The peak increase in arterial blood pressure was also similar to the FMRFamide effect. The pentapeptide was tested on rat brain stem neurones. Depending on which area of the brain stem the pentapeptide was applied to, its action was either inhibitory or excitatory. Its actions on these neurones corresponded to those of FMRFamide.

Two FMRFamide related peptides have been structurally characterized by Yang et al, (1985) from bovine brain extracts, one an octapeptide and the other an octadecapeptide. These bovine neuropeptides, along with the chicken brain pentapeptide were tested in a RIA and two standard bioassays (Greenberg et al, 1988). The effects were relatively weak but nevertheless similar to those of FMRFamide. The chicken brain pentapeptide was the most potent of the three. This was not surprising since it had the closest structural similarity to FMRFamide.

1.6.9 RELEASE OF FMRFamide RELATED PEPTIDES

Studies of neuropeptide release from invertebrate neurones is difficult because of the small amounts involved. However, as has been shown with Helisoma important results are now forthcoming.

Release of FMRFamide from Macrocallista ganglia was detected using RIA by Nagle, (1982). It occurred

upon depolarizing the preparation with a high K^+ containing saline and was Ca^{2+} dependent.

FMRFamide-like immunoreactive release from neurohaemal organs of the moth Manduca sexta was analysed by Carroll et al, (1986). When these organs were placed in normal saline there was a basal release. This was doubled in the presence of a high K^+ , Ca^{2+} containing saline. Little FMRFamide immunoreactivity was released in a high K^+ , Ca^{2+} free saline. It was therefore dependent upon depolarization and the presence of Ca^{2+} .

FMRFamide-like immunoreactive release from Helix ganglia was shown to occur by exposing the preparation to a high K^+ containing saline (Lesser et al, 1989). It was also dependent on Ca^{2+} levels.

1.7 ACTIONS AND ROLES OF FMRFamide RELATED PEPTIDES ON MOLLUSCAN NEURONES

The large, readily identifiable neurones found in the nervous systems of certain molluscan species have been utilized to great effect in studies of the actions and roles of the FMRFamide related peptides in nervous tissue. Examples of such studies under the headings of the species used and currents examined are given below. In some cases the intracellular mechanisms underlying the actions of these neuropeptides have been elucidated. A summary of the details given in this section are shown in Table 1.3.

1.7.1 HELIX

Cottrell et al, (1984) and Cottrell and Davies (1987) tested the effects of FMRFamide and some related peptides on identified neurones of Helix aspersa. Some were hyperpolarized and others depolarized. A combination of these two effects occurred in some neurones.

1.7.1.1 SLOW HYPERPOLARIZING K⁺ CURRENT (SLOW IK⁺)

FMRFamide application to the C1 neurone of each cerebral ganglion and several suboesophageal ganglia neurones induced a hyperpolarizing response which involved an increase in membrane conductance. The response did not desensitize with repeated applications and the reversal potential (-70 to -75mV) indicated that it might be due to the production of an outward K⁺ current. This was confirmed by ion substitution experiments and the reduction of the response by K⁺ channel blockers such as tetraethylammonium (TEA) and intracellular cesium ions (Cs⁺). FLRFamide and the synthetic tetrapeptide Phe-Ile-Arg-Phe-NH₂ (FIRFamide) both induced a similar response in the C1 neurone although FIRFamide was slightly less potent. The implication was that all three neuropeptides acted on the same receptor.

1.7.1.2 FAST HYPERPOLARIZING K^+ CURRENT (FAST IK^+)

The synthetic heptapeptide Tyr-Gly-Gly-Phe-Met-Arg-Phe-NH₂ (YGGFMRFamide) and pQDPFLRFamide hyperpolarized a number of identified neurones which included F76, F77. This response did not desensitize with repeated applications. It was found to be due to the production of an outward K^+ current by the same means used to study the slow K^+ current. The response induced by the heptapeptides was considerably faster than that of tetrapeptides, hence the terms "fast" and "slow". The fast response was not observed on the C1 neurone and application of high concentrations of the heptapeptides induced only a slow K^+ current. The differences between the fast and slow responses suggested that they may have been due to the activation of two different receptors.

1.7.1.3 DEPOLARIZING, VOLTAGE DEPENDENT DECREASE OF A K^+ CURRENT (DECREASED $IK^+(v)$)

When the C1 neurone was held at potentials between -40 and -20mV, FMRFamide induced a biphasic response which was comprised of an outward slow K^+ current and an inward current (Cottrell, 1982). Depolarizing the neurone even further allowed the inward current to predominate. YGGFMRFamide and pQDPFLRFamide were more potent than the tetrapeptides at producing the effect (Cottrell et al, 1984). This current was found to be

due to the reduction of an outward K^+ current which showed some Ca^{2+} dependency.

Single channel recordings using cell attached patches showed that application of FnLRFamide (a synthetic FMRFamide analogue) or YGGFMRFamide reduced the number of channel openings in a depolarized patch (Cottrell et al, 1984). The channels closed were K^+ channels. The neuropeptides had no direct access to the patch of membrane under investigation. Therefore, the neuropeptide would have had to generate an intracellular second messenger in order to influence the activity of the patch.

5-HT application to the depolarized C1 neurone also reduces an outward K^+ current. It was shown that 5-HT and the FMRFamide related peptides might operate on the same K^+ channels since FMRFamide had no effect in the presence of 5-HT (Davies, 1986). Studies of the intracellular events which underlie the effect of 5-HT have indicated that protein kinase C activation and protein phosphorylation are involved (Hill-Venning and Cottrell, 1988; Cohen et al, 1989).

1.7.1.4 FAST DEPOLARIZING Na^+ CURRENT (FAST INa^+)

FMRFamide application to the E13 neurone of the visceral ganglion induced a fast depolarization which desensitized rapidly. This depolarization was due to the influx of Na^+ . The heptapeptides pQDPFLRFamide and YGGFMRFamide were unable to produce a response (Cottrell and Davies, 1987).

Recently, this response has been studied in more detail by Cottrell et al, (1990). The F2 and C2 neurones also responded to FMRFamide with a fast Na^+ inward current. Combined data from the F2 and C2 neurones were presented. The response in the F2 neurone was unaffected by $100\mu\text{M}$ tubocurarine which at this concentration effectively abolishes the fast depolarizing responses to ACh, dopamine, histamine, aspartate and glutamate in molluscan neurones (Carpenter et al, 1977). When FMRFamide was applied to outside out patches excised from isolated or in situ C2 neurone cell bodies, small unitary currents were observed at negative potentials. Often more than one channel was observed which indicated that the FMRFamide receptors are clustered. Less than 10% of the patches obtained responded to FMRFamide suggesting a sparse distribution of receptors. The response current/voltage relationship of these unitary currents was in agreement with a pure Na^+ conductance. The unitary currents were still seen when the patches had been isolated for 20 minutes which indicated that a second messenger was not required for the response. This is the first known example of a neuropeptide directly activating a ligand gated ion channel.

1.7.1.5 RECEPTORS

Cottrell and Davies (1987) interpreted these four responses of the FMRFamide related peptides in terms of the activation of four different receptor types. They

found that the C-terminal amino acid sequence of Phe-Met (or Leu)-Arg-Phe-NH₂ was required by these receptors for them to be activated. Analogues with amino acid substitutions in any of these positions or the amide group removed had no effect on the Helix neurones tested.

1.7.1.6 RELATED STUDIES ON K⁺ AND Na⁺ CURRENTS

Boyd and Walker (1985; 1987) also examined the actions of FMRFamide related peptides on identified Helix neurones. Their results generally corresponded with Cottrell's group. FMRFamide and FLRFamide induced hyperpolarizing outward K⁺ currents and depolarizing inward Na⁺ currents, although FLRFamide was much less potent. pQDPFLRFamide hyperpolarized neurones with varying potencies but did not produce excitation in any of the neurones tested. FMRFamide and pQDPFLRFamide were both quoted as hyperpolarizing the E13 neurone. This was contrary to the findings of Cottrell's group who report that FMRFamide depolarized the E13 neurone and pQDPFLRFamide had no effect.

1.7.1.7 Ca²⁺ CURRENTS (DECREASED I_{Ca}²⁺(v) INCREASED I_{Ca}²⁺(v))

In two identified Helix aspersa neurones, D3 and E2, FMRFamide decreased the duration of the Ca²⁺ action potential (Colombaioni et al, 1985). In another neurone, the E11, FMRFamide decreased Ca²⁺ conductance,

the amplitude of the Ca^{2+} spike plateau and a cyclic AMP dependent K^+ conductance known as the "S" K^+ current.

The effects of FMRFamide related peptides on a Ca^{2+} current of the C1 neurone were studied by Cottrell and Lesser, (1987). FMRFamide and FLRFamide reversibly reduced the total inward Ca^{2+} current by 30%. pQDPFLRFamide and YGGFMRFamide had no consistent inhibitory effect on the current. The fact that the tetrapeptides had an effect whilst the N-terminally extended heptapeptides did not, is a similar situation to that described with the fast Na^+ current (Cottrell and Davies, 1987).

It has been shown by Yakel, (1991) that FMRFamide can both inhibit and enhance the Ca^{2+} current in cultured Helix central neurones via independent mechanisms. The inhibition usually involved a decrease in amplitude and rate of inactivation of the current. Pertussis toxin (an inhibitor of G_i and G_o proteins) produced a time dependent block of both the FMRFamide induced decrease in amplitude and decrease in the rate of inactivation of the Ca^{2+} current. The irreversible activation of G proteins by GTP γ S did not reduce the amplitude of the current but did reduce the inactivation rate, the latter effect being a mimic of the FMRFamide response. There was no evidence to indicate that any of the commonly known second messenger systems were involved. These results suggested that the inhibition of the Ca^{2+} current may have been due to a direct coupling between the

FMRFamide receptors and Ca^{2+} channels through a pertussis toxin sensitive G protein. The FMRFamide induced enhancement of the Ca^{2+} current reduced with time and the inclusion of NAD^+ in the patch electrode filling solution, diminished this effect, suggesting that a NAD^+ dependent intracellular mechanism could modulate the response. Ca^{2+} current enhancement was not associated with changes in the rate of activation or inactivation. The enhancement does not appear to involve a pertussis toxin sensitive G protein.

1.7.2 APLYSIA

1.7.2.1 K^+ CURRENTS (INCREASED $\text{IK}^+(\text{s})$)

FMRFamide and 5-HT have opposing effects on the electrical excitability of Aplysia sensory neurones and on transmitter release from sensory neurone presynaptic terminals (Siegelbaum et al, 1982; Belardetti et al, 1987). These effects could be termed as modulatory. FMRFamide produced an increase in probability that a K^+ channel known as an "S" K^+ channel is opened. This results in a slow, inhibitory hyperpolarization and a decrease in action potential duration which contributes to presynaptic inhibition. In contrast, 5-HT produced prolonged, all or nothing closure of the "S" K^+ channels by acting through cyclic AMP dependent protein phosphorylation. This results in a slow depolarization and an increase in the duration of the action potential which contributes to presynaptic facilitation.

Furthermore, FMRFamide could override the excitatory action of 5-HT at the single channel level so that FMRFamide reopens "S" K^+ channels closed by 5-HT or cyclic AMP.

A number of studies were performed in an attempt to elucidate the intracellular mechanisms underlying these actions of FMRFamide (Piomelli et al, 1987). Application of arachidonic acid to sensory neurones mimicked the effects of FMRFamide. The 12-lipoxygenase metabolite of arachidonic acid, 12-hydroperoxyeicosatetraenoic acid (12-HPETE), also mimicked the action of FMRFamide. It was thought that this metabolite of arachidonic acid was produced intracellularly leading to the opening of the "S" K^+ channels. However, the situation is complicated in that 12-HPETE could directly modulate "S" K^+ channels when applied to the outside membrane of outside out patches (Buttner et al, 1989). There is a possibility that this metabolite can interact with a site on the channel protein near the outer surface of the membrane.

Volterra and Siegelbaum, (1988) looked at how the action of FMRFamide on these neurones could be linked to the production of arachidonic acid. Using activators and inhibitors of G proteins they concluded that a pertussis toxin sensitive G protein was most likely to couple the FMRFamide receptor to phospholipase activation and arachidonic acid production.

Sweatt et al, (1989) examined the combined effects of FMRFamide and 5-HT on protein phosphorylation of these neurones. FMRFamide decreased the levels of

protein phosphorylation without altering the level of cyclic AMP. FMRFamide also overrode the cyclic AMP mediated enhancement of transmitter release produced by 5-HT and reversed the cyclic AMP dependent increase in protein phosphorylation produced by 5-HT. It was unclear whether the reduction of protein phosphorylation was caused by protein kinase inhibition or phosphatase activation.

Ichinose and Byrne (1991) looked at the role of protein phosphatases in the opening and closure of "S" K^+ channels by FMRFamide and 5-HT. The protein phosphatase 1 and 2A inhibitor, okadaic acid, and purified protein phosphatase enzymes were used. They found that in the absence of physiological stimulation, basal levels of phosphorylation regulated the channel. The FMRFamide, 5-HT and cyclic AMP induced responses were regulated by protein phosphatases and the FMRFamide induced outward "S" K^+ current may be partly due to the activation of protein phosphatases.

In a recent study by Shi and Belardetti, (1991) the effects of 5-HT, cyclic AMP and FMRFamide on sensory neurones were examined in detail. They found that application of $100\mu M$ FMRFamide in the presence of membrane soluble 8-bromo cyclic AMP resulted in an outward current larger than the control FMRFamide response and equal to the sum of 8-bromo cyclic AMP and FMRFamide alone. When FMRFamide was applied at $500\mu M$ it completely antagonized the closing actions of maximal 8-bromo cyclic AMP ($100\mu M$). This confirmed in these neurones that FMRFamide can open "S" K^+ channels closed

by cyclic AMP. However, with moderate concentrations of 5-HT, which produce a slow inward current due to closures of the "S" K^+ channels, FMRFamide could only partially antagonize this action. At maximal 5-HT concentrations ($0.1\mu M$) 5-HT response was not antagonized by any FMRFamide concentration and FMRFamide responses were smaller than control. These results indicated that 5-HT could inhibit the FMRFamide response by a cyclic AMP-independent mechanism. They also confirmed that FMRFamide responses could be mimicked by the application of arachidonic acid.

Brezina et al, (1987a) found that FMRFamide and YGGFMRFamide were equally effective in activating an outward K^+ current in abdominal ganglion neurones of Aplysia (R2, L2, L3, L4, and L6). The current was independent of voltage and Ca^{2+} levels. It was blocked by 5-HT and raised cyclic AMP levels. Patch clamp studies showed that channel activity was reduced by 5-HT and enhanced by FMRFamide. The evidence indicated that this was a "S" K^+ current. Brezina, (1988) later found that GTP γ S and ACh activated outward K^+ currents which were identical to the FMRFamide induced current. It was thought that FMRFamide and ACh, acting through different receptors, activated the same K^+ current by a mechanism involving a G protein.

FMRFamide has also been shown to have effects on the duration of action potentials, excitability and membrane currents in tail sensory neurones of Aplysia (Critz et al, 1991). Whereas 5-HT increased the duration of action potentials and caused an enhancement

of excitability in cell bodies of tail sensory neurones, FMRFamide reversed these actions and decreased the duration of excitability and action potentials. It was found that FMRFamide had actions on three K^+ currents. One current which was increased by FMRFamide had properties indicating it was an "S" K^+ current. Another current reduced by FMRFamide was a Ca^{2+} dependent K^+ current. A third current increased by FMRFamide with large depolarizations appeared to be a delayed or voltage dependent K^+ current. Extracellular application of arachidonic acid mimicked the action of FMRFamide on the three K^+ currents which indicated that this second messenger may be underlying these effects.

1.7.2.2 Na^+ CURRENTS ($I_{Na^+}(v)$)

Ichinose and McAdoo, (1988) characterized the effects of FMRFamide on the R14 abdominal ganglion neurone of Aplysia californica and Aplysia brasilina. FMRFamide induced a fast outward current, a fast inward current and a slow inward current. The slow inward current was examined in detail. It was carried mainly by Na^+ and exhibited voltage dependency. The largest amplitude occurred between -40 and -20mV. The amplitude of the current was independent of external K^+ but did increase when external Ca^{2+} and Cl^- concentrations were reduced. The current also desensitized upon successive applications of FMRFamide.

Ichinose and McAdoo, (1989) discovered that by injecting cyclic GMP into the R14 neurone they could

mimic the FMRFamide response. The cyclic GMP current had the same characteristics of the FMRFamide response described above. Phosphodiesterase inhibitors which prevent the metabolism of cyclic nucleotides increased the cyclic GMP and FMRFamide induced inward current. These results indicated that FMRFamide opens the Na^+ channels by activating the cyclic GMP intracellular second messenger system.

1.7.2.3 Ca^{2+} CURRENTS (DECREASED $\text{I}_{\text{Ca}^{2+}(\text{v})}$)

Brezina et al, (1987b) showed that FMRFamide and YGGFMRFamide were equally effective in suppressing a pharmacologically isolated Ca^{2+} current in abdominal ganglion neurones (R2, R15, L2, L3, L4 and L6) of Aplysia. This suppression was incomplete suggesting that more than one class of Ca^{2+} channel was present only, one of which was neuropeptide sensitive. The rate of activation and inactivation of the remaining Ca^{2+} current was unaffected. The intracellular second messenger systems involving cyclic AMP, cyclic GMP, Ca^{2+} , IP_3 and protein kinase C activation did not appear to be involved in the suppression. The suppression did seem to be mediated by the same receptor as that causing the outward "S" K^+ current. The Ca^{2+} current effect and the "S" K^+ current were both mimicked by GTP γ S injection implicating the involvement of G proteins.

1.7.2.4 Cl⁻ CURRENTS (ICl⁻(v))

When FMRFamide was applied to the L2 abdominal ganglion neuron of Aplysia, it activated an inwardly rectifying current and reduced the amplitude of the transient K⁺ current (Thomson and Ruben, 1988). The reversal potential of the inward rectifier tended to be variable but was near -40mV at the start of experiments. The current was voltage dependent. Changes in external Na⁺, Ca²⁺, or K⁺ concentrations had no effect but lowering the external Cl⁻ concentration had complicated effects on the current amplitude. The current was also dependent on intracellular Cl⁻ concentrations. It was concluded that the inward rectifier was a Cl⁻ current.

1.7.3 HELISOMA

The characteristics of the peptidergic synapse formed between the FMRFamide related peptide containing VD4 (presynaptic) and P1 (postsynaptic) neurones have already been described in Section 1.6.7. Here attention will be drawn to studies performed by Man-Son-Hing et al, (1989) and Haydon et al, (1991).

Man-Son-Hing et al, (1989) looked at the cholinergic chemical synapse formed in cell culture by Helisoma neurones B5 and B19. It was possible to depolarize the B5 neurone to evoke transmitter release and record the postsynaptic currents in the B19 neurone. When FMRFamide was applied, the number of

depolarization evoked postsynaptic currents were reduced although the postsynaptic sensitivity to ACh was unaffected. Therefore FMRFamide was probably causing presynaptic inhibition of ACh release from the B5 neurone.

By voltage clamping the B5 neurone they found that FMRFamide reduced the magnitude of the high voltage activated Ca^{2+} (decreased $\text{I}_{\text{Ca}^{2+}(\text{v})}$) current which raises intracellular Ca^{2+} and triggers ACh release. The Ca^{2+} dependent secretory apparatus of the B5 neurone was also examined. The elevation of intracellular Ca^{2+} levels to evoke transmitter release was achieved by injecting the photolabile Ca^{2+} cage, nitr-5. This dissociated the voltage dependent current from the Ca^{2+} dependent secretory event and in a sense was a Ca^{2+} clamp. Exposure to ultraviolet light released Ca^{2+} from the cage and this increased the rate of postsynaptic currents in the B19 neurone which was due to ACh release. FMRFamide application reversibly reduced the rate of the postsynaptic current. The level of free Ca^{2+} in the B5 neurone was unaffected by FMRFamide. Therefore, the suggestion was that FMRFamide somehow reduced the sensitivity of the secretory apparatus to elevated internal Ca^{2+} . This was the first report of such an effect of FMRFamide or any other neuropeptide on neurones.

In a more recent study by Haydon et al, (1991), it was found that FMRFamide modulation of secretory machinery underlying presynaptic inhibition of synaptic transmission in cultured Helisoma neurones, requires a

pertussis toxin sensitive G protein. The synapse examined was again that between neurones B5 and B19. Neurotransmitter release at the cultured synapses was found to be quantal in nature. FMRFamide application reduced the frequency of miniature inhibitory postsynaptic currents (MIPSCs) without affecting free intracellular Ca^{2+} concentration. GTP γ S injection into the presynaptic neurone mimicked the effect of FMRFamide. When pre-activated pertussis toxin was injected into the presynaptic neurone, FMRFamide was unable to reduce either MIPSC frequency or the magnitude of action potential evoked inhibitory postsynaptic currents. It was found that concentrations of less than 10^{-7}M FMRFamide was able to modulate the secretory machinery, a concentration that does not effect Ca^{2+} current amplitude.

1.8 AIMS AND OBJECTIVES

The examples quoted show that neuropeptides influence a variety of ionic currents in both vertebrate and invertebrate neurones. The general procedure has been to characterize these actions electrophysiologically and pharmacologically at the outset and then attempt to understand the mechanisms involved in coupling the receptors to the ion channels. A combination of these approaches have been used in the work presented in this thesis to examine two currents induced by FMRFamide in identified neurones of Helix aspersa. The aims were: 1) to elucidate the

Table 1.3

Summary of the FMRFamide related peptide effects on molluscan neurones described in the text. The mechanisms of action of the neuropeptides are listed where appropriate.

Slow IK^+	<u>Helix</u> C1 neurone. FMRFamide, FLRFamide more potent than FIRFamide. Mechanism studied in this thesis.
Fast IK^+	<u>Helix</u> F76, F77 neurones. pQDPFLRFamide, YGGFMRFamide. Mechanism unknown.
Decreased $IK^+(v)$	<u>Helix</u> C1 neurone. pQDPFLRFamide, YGGFMRFamide more potent than FMRFamide. 5-HT has same effect by activating protein kinase C
$IK^+(s)$	<u>Aplysia</u> sensory neurones. FMRFamide. Mechanism through PTX sensitive G protein, lipoxygenase metabolites of arachidonic acid. Protein phosphorylation involved.
$IK^+(s)$	<u>Aplysia</u> R2, L2, L3, L4 and L6 neurones. FMRFamide, YGGFMRFamide. Mechanism involves a G protein.
$IK^+(s)$	<u>Aplysia</u> tail sensory neurones. FMRFamide. Arachidonic acid involved.
$IK^+(Ca^{2+})$	<u>Aplysia</u> tail sensory neurones. FMRFamide. Arachidonic acid involved.
$IK^+(v)$	<u>Aplysia</u> tail sensory neurones. FMRFamide. Arachidonic acid involved.
Fast INa^+	<u>Helix</u> C2, F2 and E13 neurones. FMRFamide. Mechanism is a ligand gated ion channel.
$INa^+(v)$	<u>Aplysia</u> R14 neurone. FMRFamide. Mechanism through cyclic GMP pathway.
Decreased $ICa^{2+}(v)$	<u>Helix</u> C1, D3 and E2 neurones. FMRFamide, FLRFamide. Mechanism unknown.
Decreased $ICa^{2+}(v)$	<u>Aplysia</u> R2, R15, L2, L3, L4 and L6 neurones. FMRFamide, YGGFMRFamide. Mechanism through a G protein. Not through cyclic AMP, cyclic GMP, Ca^{2+} , IP_3 or protein kinase C.
Decreased $ICa^{2+}(v)$	<u>Helix</u> cultured central neurones. FMRFamide. Mechanism: receptors coupled directly to a PTX sensitive G protein.

Decreased $\text{ICa}^{2+}(\text{v})$	VD4 and P1 neurones in culture. FMRFamide, FLRFamide and GDPFLRFamide. G protein activation involved. VD4 stimulation mimicked peptide action.
Increased $\text{ICa}^{2+}(\text{v})$	<u>Helix</u> cultured central neurones. FMRFamide. NAD^{+} dependent mechanism.
$\text{ICl}^{-}(\text{v})$	<u>Aplysia</u> L2 neurone. FMRFamide. Mechanism unknown.
* Unique effect	<u>Helisoma</u> cultured B5 and B19 neurones. FMRFamide. Decreased Ca^{2+} sensitivity of secretory apparatus. Modulation of secretory apparatus through activation of pertussis toxin sensitive G protein.

mechanism(s) underlying the FMRFamide slow K^+ current in the C1 neurone and 2) to begin to characterize the FMRFamide fast Na^+ current in the C2 neurone.

Chapter 2

Methods

2.1 THE EXPERIMENTAL PREPARATION

2.1.1 ANIMAL MAINTENANCE

Helix aspersa were collected locally and upon arrival in the laboratory were in a state of hibernation. Inactive animals were activated by placing them in a shallow volume of warm water (30-35°C). Once activated, the animals were maintained in this state at room temperature (18-22°C) by placing them in a moist environment which consisted of damp tissue paper within a large, sealed, plastic container with air holes. This container also allowed sufficient light penetration. Fresh lettuce leaves were provided as a food source.

2.1.2 DISSECTION

All experiments were performed on C1 and C2 neurones of the cerebral ganglia.

An active snail was decapitated using scissors at a point shown in Figure 2.1 and the head pinned out on a wax dissecting board. One pin was inserted through the mouth and another through the oesophagus. When the skin was cut and pulled back, the position of the circumoesophageal complex of ganglia (cerebral ganglia and suboesophageal ganglia) was revealed. This was removed from the animal and placed in a small Perspex bath (1ml) which was lined with a Sylgard base (Sylgard 184 silicone elastomer, Dow Corning) and filled with normal Helix saline (80mM NaCl, 5mM KCl, 5mM MgCl₂, 7mM

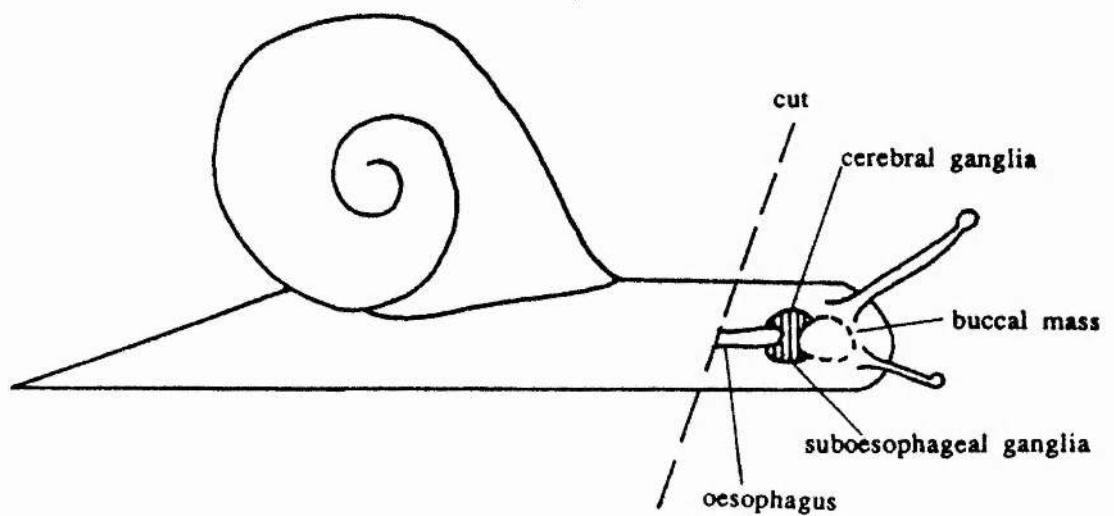


Figure 2.1

Diagram showing where the head of an activated snail was decapitated and the relative positions of the cerebral and suboesophageal ganglia within the head. The cerebral ganglia lie in the dorsal surface of the oesophagus and the suboesophageal ganglia on the ventral surface.

CaCl₂, 20mM Hepes, pH 7.5). From this point all manipulations of the tissue were carried out with the aid of a binocular dissecting microscope (Nikon) at magnifications ranging from x9 to x40. A tapered glass rod attached to a light source was placed close to the tissue inside the bath to illuminate the preparation.

The cerebral and suboesophageal ganglia were separated by cutting through the nerve connectives using fine dissecting scissors. The cerebral ganglia were pinned out on the Sylgard base with the ventral surface uppermost. The cell bodies of the neurones are normally covered with two layers of connective tissue. The outer layer was removed using two pairs of fine forceps. The inner layer of connective tissue is transparent, thin and very delicate. Great care was required to rupture this layer with forceps and expose the cell bodies of the individual neurones. The relative positions of the C1 and C2 neurones are shown in Figure 2.2. If the exposed neurones were required for patch clamping experiments, they were bathed in normal Helix saline containing 0.1% (w/v) trypsin (Sigma) for 15 minutes after which they were washed thoroughly with normal Helix saline. The enzyme trypsin was used to make it more suitable for high resistance seals to be obtained between the patch pipettes and neuronal cell membrane.

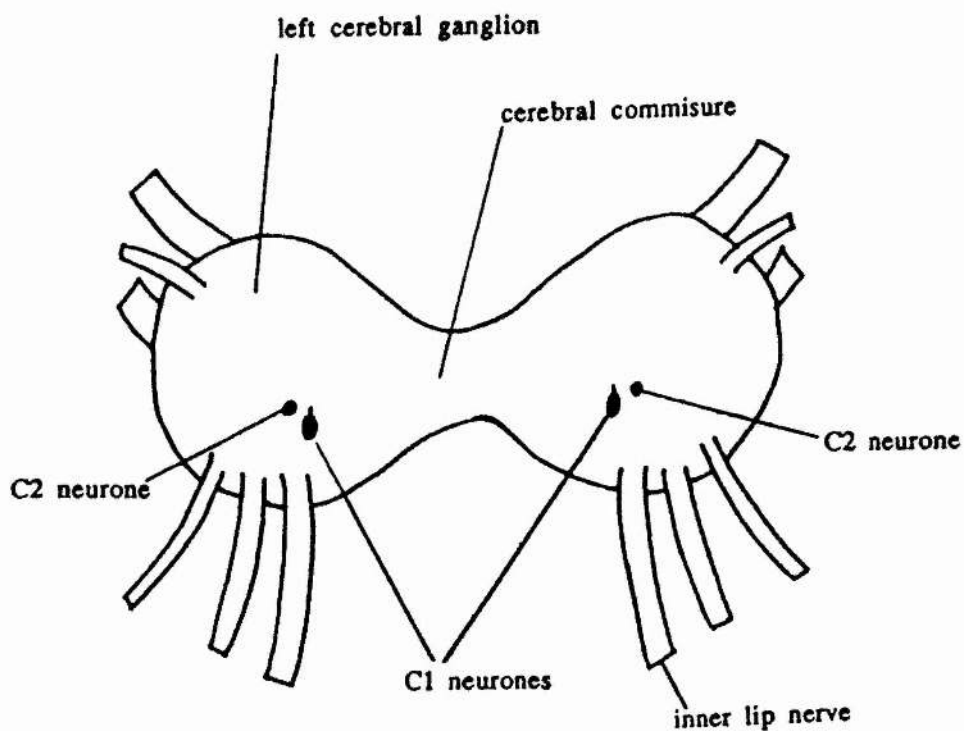


Figure 2.2

A simplistic illustration of the cerebral ganglia as they were pinned out in the bath with the ventral surface uppermost. The relative positions of the C1 and C2 neurones are shown.

2.2 PERFUSION SYSTEM

An important requirement for most of the experiments was to have a perfusion system available. This meant that as well as being able to provide a constant flow of saline across the preparation, salines containing membrane soluble analogues of second messengers, enzyme activators, enzyme inhibitors and varying ionic concentrations could also be applied to the neurones.

The system consisted of various reservoirs (50ml syringes) connected to a multiwell tap via separate lines of soft plastic tubing. The outflow of the tap led to the small Perspex bath containing the preparation. By switching the tap to the appropriate reservoir, different salines could be brought into the perfusion stream. The driving force necessary to allow the perfusate to flow was provided in that the reservoirs were about 50cm above the level of the bath. The flow rates from the reservoirs were controlled by adjustable screw clamps on the soft plastic tubing. The flow rate was usually set at 1ml/minute.

A suction system allowed the perfusate to be drained away from the bath. A suction tube made of plastic was connected to a collecting vessel (Buchner flask) via a drain line. The collecting vessel was attached to a small electric air pump which provided the vacuum required for suction. The suction tube was carefully positioned very close to, but just outside the bath. This ensured that as well as draining

perfusate from the bath there was always a sufficient height of solution within the bath to cover the preparation.

2.3 INTRACELLULAR RECORDING METHODS

2.3.1 RECORDING ELECTRODES

Recording electrodes were fabricated from thin walled borosilicate glass capillary tubes with internal filaments (Clark Electromedical Instruments, type GC150TF-15). The significance of the internal filament was that the electrode tips could be filled quite simply by placing a small volume of recording electrolyte solution in the end of the electrode (back-filling). The solution was dragged down to the tip by capillary action along the filament.

Prior to pulling electrodes, the ends of the glass capillary tubes were rounded in a Bunsen burner flame. This prevented sharp edges on the ends of the electrodes scraping silver chloride (AgCl) from the AgCl coated silver recording wire. Electrodes were pulled on a Narashige PP-83 two stage vertical puller and stored in Petri dishes prior to use as a protection from dust. They were back-filled with a recording electrolyte solution of 200mM KCl. The solution was delivered from a syringe and hypodermic needle incorporating a 0.22 μ m Millipore filter attachment. The electrodes when filled with this solution had a relatively low resistance, 1-2 M Ω as measured by the

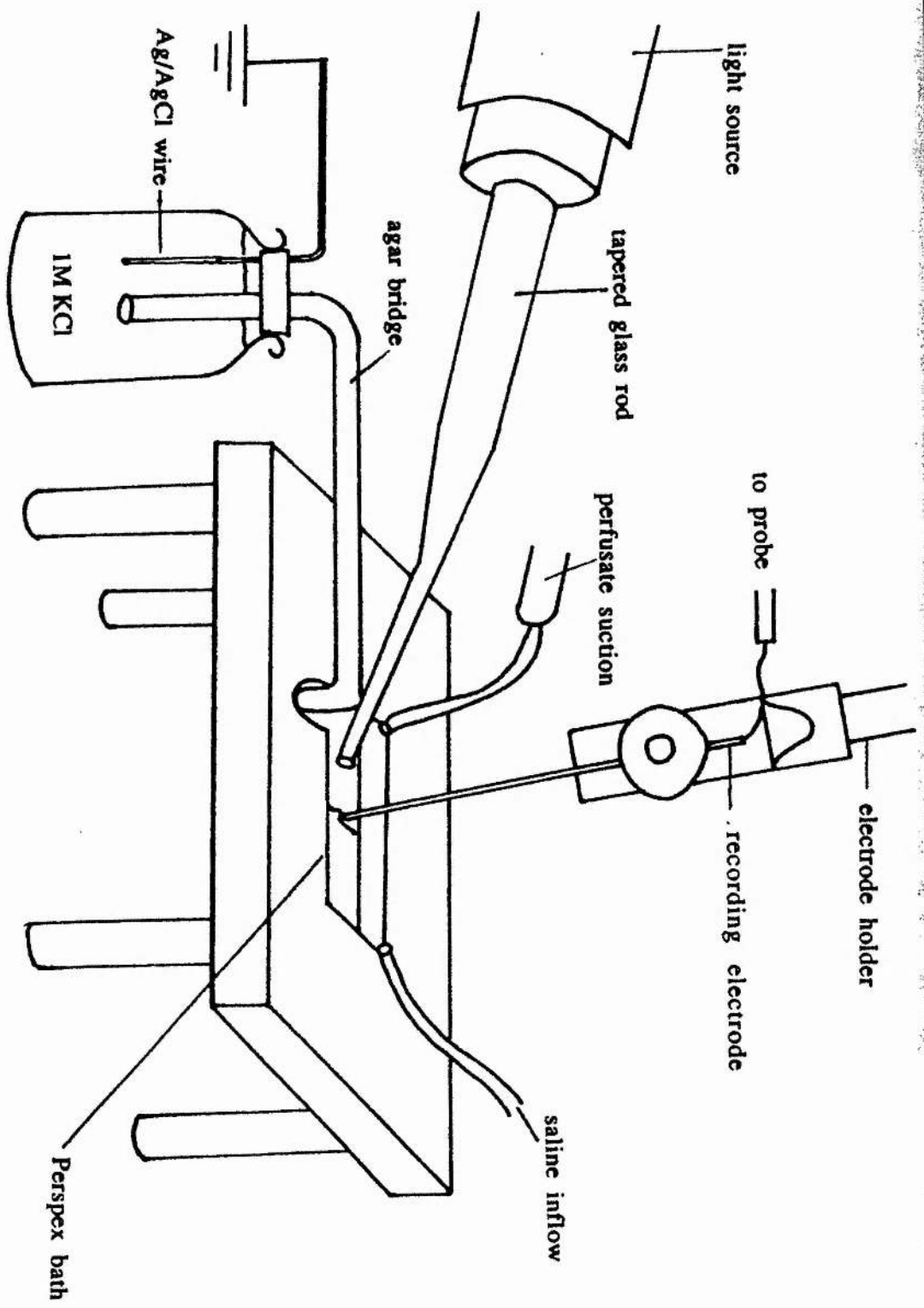
impedance facility on the Dagan 8100 single electrode voltage clamp system. The low resistance was required for voltage clamping since the electrodes must be able to pass large amounts of current.

2.3.2 RECORDING APPARATUS

Intracellular recordings from neurones were made using a Dagan 8100 single electrode voltage clamp system. Both current and voltage clamping could be performed using a single electrode. Electrodes containing 200mM KCl, mounted on a Narashige micromanipulator were connected via a AgCl coated silver wire (Ag/AgCl) to the input of the Dagan 8100 probe. Two methods were used to ground the bath containing the preparation. The first was to place a Ag/AgCl wire connected to the virtual ground of the Dagan 8100 probe into the saline solution bathing the preparation. The second involved the use of an agar bridge. This consisted of a glass tube filled with 3% (w/v) agar (BDH) made up in a 1M KCl solution. One end was placed in the bath and the other in a small bottle containing a 1M KCl solution. A Ag/AgCl wire, again connected to the virtual ground of the Dagan 8100 probe was placed in the 1M KCl solution. The agar bridge was useful because when there was significant alterations in the ionic composition of the perfusing solution, the bridge minimised changes in junctional potentials. A diagram showing the relative positions of this equipment is provided in Figure 2.3.

Figure 2.3

Side on view showing the positions of equipment around the Perspex bath which contained the preparation. A recording electrode mounted on a holder which was attached to a manipulator is the only electrode shown. On this occasion an agar bridge is demonstrated grounding the bath.



A Grass stimulator was connected to the Dagan 8100 preamplifier so that current and voltage pulses could be delivered to the neurone under investigation. The recordings of current and voltage were monitored on a three channel Tektronix 5113 oscilloscope and stored on Ampex four channel magnetic tapes using a Racal 405 tape recorder. Permanent visual records were produced by a high quality Gould 220 brush chart recorder.

2.3.3 VOLTAGE CLAMPING

Since recordings requiring high frequency responses were not under investigation in this study, it was possible to voltage clamp the neurones with a single electrode rather than a two electrode system. This reduced the complexity of the experiments performed because on certain occasions recording, iontophoresis and pressure injection electrodes were used at the same time.

When voltage clamping with the single electrode, the Dagan 8100 preamplifier was at the switched clamp/voltage setting. When at this setting the system switches at high frequency from a sensing voltage mode to a current passing mode. The switching frequency in these experiments was set at 3KHz and the duty cycle was 50%. A 50% duty cycle meant that half the time was spent in each mode.

To eliminate high frequency oscillations occurring during high gain voltage clamping, the current record was filtered before being displayed on the chart

recorder. A low pass filter made up from a 100nF capacitor and a 160k Ω resistor was used which gave a filter frequency of 9.9Hz.

2.3.4 IONTOPHORESIS

FMRFamide was applied to neuronal cell bodies by the process of iontophoresis. At neutral pH FMRFamide has a net positive charge. By applying a small positive current through a glass electrode containing a 5mM FMRFamide solution, it was possible to deliver a controlled amount of neuropeptide to the neurones. A common problem with iontophoresis was blockage of the electrode tips. This was overcome by adapting the preparation of the FMRFamide solution and fabrication of the electrodes.

Solid FMRFamide (Peninsula Laboratories) was dissolved in double distilled water. The volume was split equally between two centrifuge filter units (0.22 μ m spin x filter units, Costar U.S.A). The solutions were spun in a microfuge (Heraeus Christ) at 13000 r.p.m. for 30 seconds. Small aliquots of the filtered solutions were sucked into 1ml syringes which had short lengths of narrow plastic tubing attached. Electrodes were back-filled with neuropeptide from these syringes.

The electrodes were fabricated from the same glass type and pulled on the same puller as the recording electrodes. However, the glass capillary tubes were cut to shortened lengths to make the bodies of the

electrodes shorter. The heating element of the puller was set at a lower value on the second pull which produced wider tipped electrodes. These had resistance values of 0.5 to 0.7M Ω when filled with 200mM KCl and were less susceptible to blockages than higher resistance electrodes. Although there was a danger that these iontophoresis electrodes may have been prone to leakage, this was overcome since they had short bodies and only needed to be filled with a small volume of solution. This resulted in there being a reduced head of pressure on the electrode tips.

Filled electrodes were mounted on a hydraulically operated Narashige micromanipulator. A Ag/AgCl wire through which current could be delivered, was inserted into the FMRFamide solution. This was connected to an iontophoresis programmer which had its own direct current battery power supply. Another Ag/AgCl wire connected to the programmer acted as the grounding wire and was placed in the saline within the bath. Using this programmer in conjunction with an electronic stimulator, it was possible to set the levels of ejection current, retain current, ejection current pulse duration and the duration between the pulses. The ejection current was usually set no higher than 100nA with pulse durations between 1.5 and 2.5 seconds. A retain current of 10 to 20nA was generally used. The retain current was required to prevent any leakage of FMRFamide from the electrode tip. It had the opposite polarity (negative) to the ejection current and was

applied constantly apart from when FMRFamide was ejected.

2.3.5 PRESSURE INJECTION

Due to the membrane insolubility of certain compounds used in some experiments on the C1 neurone, it was necessary to inject them directly into the cell body. This was achieved by applying controlled pulses of compressed air to a solution contained within an electrode using a Picospritzer unit (General Valve U.S.A.).

The electrodes used for pressure injection were identical to the iontophoresis electrodes. All of the solutions used in these electrodes were passed through $0.22\mu\text{m}$ (Millipore) filters in order to prevent blockages in the tips. Fast green dye at a concentration of 1% (w/v) was also included in the solutions so that successful injections could be visually verified.

A Ag/AgCl wire connected to a probe for a Neurolog preamplifier was incorporated into the airtight holder for pressure electrodes. This was used to provide a definite monitor of pressure electrode impalement of a neurone. The voltage signal from this preamplifier was displayed on the third channel of the Tektronix 5113 oscilloscope.

2.3.6 DILUTION FACTOR CALCULATION

Injection of a solution into the cell body of a neurone caused it to be diluted in the cytoplasmic fluid volume. The magnitude of this dilution depended upon the volume of solution injected from the electrode and the volume of the cell body.

The volume of solution injected from a pressure electrode was quantified as follows. A pressure electrode filled with 1% (w/v) fast green dye solution made up in distilled water was placed in the electrode holder and manoeuvred into the Perspex bath which contained microscope immersion oil (Zeiss). An ejection was then carried out at a pressure pulse of 138kPa for 10ms. These values for air pressure and pulse duration were typical for those used in experimental situations. The dyed distilled water formed a sphere in the immersion oil, the diameter of which could be measured using a microscope eyepiece micrometer. This value was used to calculate the volume (v) of the sphere and hence the volume of solution ejected using the formula $v = 4/3\pi(\text{radius})^3$. This procedure was carried out on ten occasions with the average volume ejected being 1×10^{-12} litres (1pl).

The volume of the C1 neurone cell body was estimated again by measuring its diameter and basing the calculation on the volume of a sphere. The neuronal cell body was not in a perfect spherical form. However, when the volume of the C1 neurone cell body isolated in culture situations was measured, it did form a more

perfect spherical shape and the value was similar to that of the in situ neurone. The volume of the cell body was calculated at an average of 1×10^{-9} litres (1nl). Therefore, the dilution factor of a solution injected into the cell body of the C1 neurone was approximately 1000 fold.

2.3.7 ARRANGEMENT OF EQUIPMENT

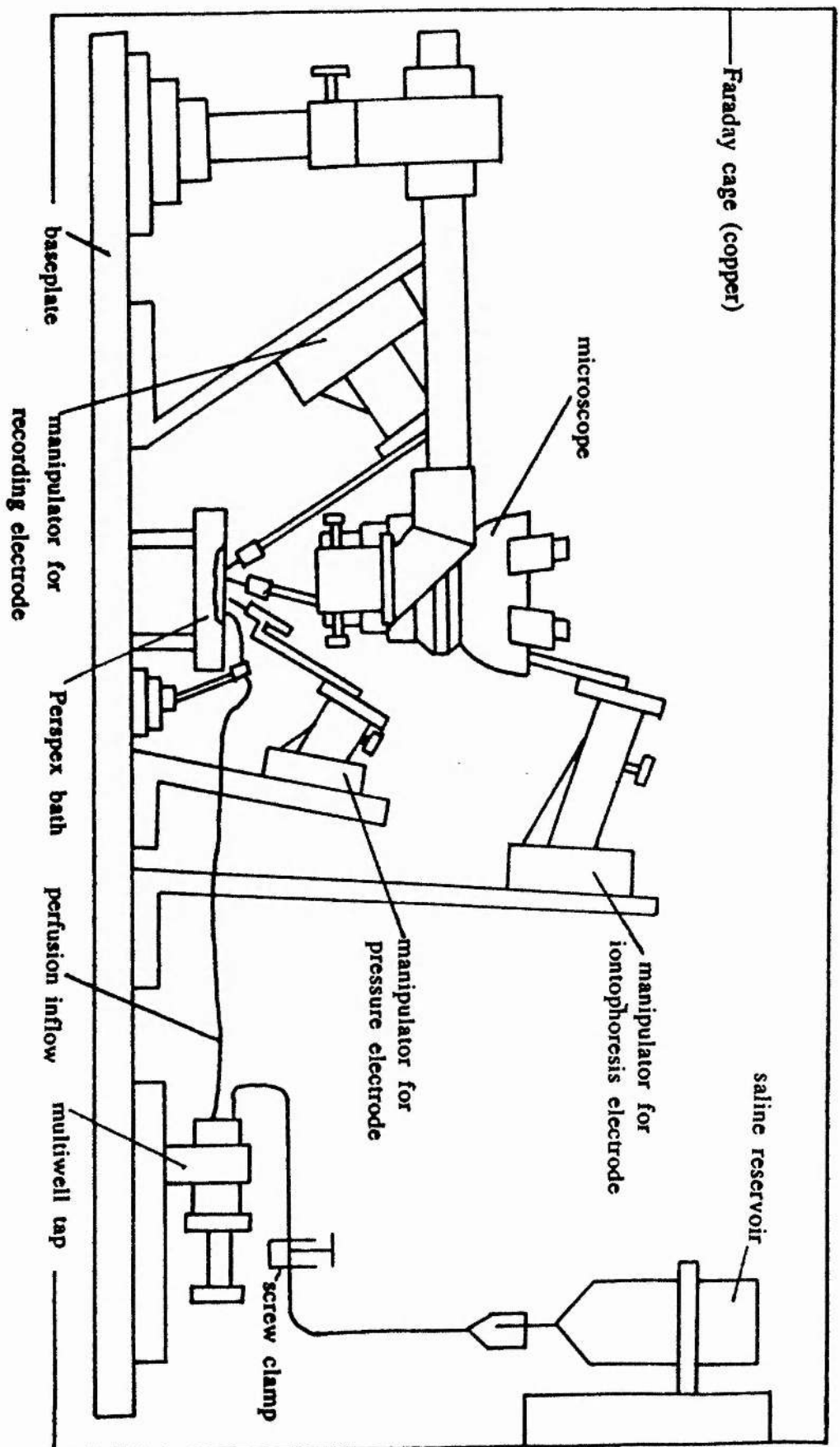
The Perspex bath from which recordings were made, perfusion system, light source, micromanipulators, preamplifier probes and the dissecting microscope were all arranged on a baseplate within a copper Faraday cage (simplified diagram shown in Figure 2.4). All pieces of equipment which could have acted as aerials for electrical noise were connected to a common ground along with the baseplate and Faraday cage. The ground used was that of the Grass stimulator. The electronic apparatus was arranged on a rack mounting next to the cage.

2.3.8 ELECTROPHYSIOLOGICAL PREPARATION OF A NEURONE PRIOR TO EXPERIMENTATION

The steps outlined below describe the preparation necessary for an experiment where pressure injection of a solution was required. However, in all other experiments where voltage clamping and neuropeptide application were used, the same procedure was carried out, minus the pressure electrode.

Figure 2.4

The arrangement of equipment used for intracellular recording experiments is represented in this diagram. The steel baseplate and copper Faraday cage were grounded to reduce electrical noise. The suction system used to draw perfusate from the bath and the agar bridge are not shown for the sake of simplifying the diagram.



With the cell body having been exposed, the tip of the recording electrode was placed in the bath saline using a Narashige micromanipulator. In current clamp mode the resistance of the electrode was checked. It was then moved close to the cell body. When the electrode actually touched the cell membrane this registered as a slight hyperpolarization on the voltage trace observed on the oscilloscope. The neurone was carefully impaled using the fine controls of the micromanipulator. The immediate level of hyperpolarization was a measure of the resting membrane potential of the neurone. The neurone was then actively hyperpolarized by injecting negative current from the Dagan through the electrode. This helped the neurone to recover from the original trauma of impalement. The perfusion of saline was then started which also aided neurone recovery.

An iontophoretic electrode containing FMRFamide solution was placed into the bath using a hydraulically operated Narashige micromanipulator. Once in the bath saline, the iontophoresis programmer was switched on and a retain current applied to the FMRFamide solution. An ejection current was applied to check if the electrode was free from any possible blockage. The electrode was then moved close ($10-100\mu\text{m}$) to the cell body.

A pressure injection electrode mounted on an air tight holder was manoeuvred into the bath saline using a third Narashige micromanipulator. An air pressure test pulse was passed through the electrode checking

for uninhibited flow of dyed solution from the tip. The Neurolog preamplifier was switched on and the voltage signal from this monitored on the oscilloscope. The neurone was impaled by careful operation of the fine controls of the manipulator. Impalement of the neurone resulted in this voltage signal moving in the hyperpolarizing direction. However, the overall effect of pressure electrode impalement was a depolarization of the neurone resulting in spiking. More hyperpolarizing current was delivered through the recording electrode from the Dagan to aid the recovery of the neurone from this second impalement. The pressure electrode was withdrawn very slightly from its original position within the cell body. This ensured that the electrode tip was not compressed against the nucleus or opposite side of the cell body, hence avoiding blockage.

With all three electrodes in position the Dagan was switched to voltage clamp mode and the experiment could begin.

2.4 PATCH CLAMP METHODS

2.4.1 PATCH PIPETTES

The fabrication process for patch pipettes consisted of a number of steps which have been described by Hamill et al (1981) with a few modifications.

Patch pipettes were made from thin walled borosilicate glass capillary tubes (Clark Electromedical Instruments, type GC150T-15). Prior to pulling pipettes, the ends of the glass capillary tubes were rounded in a Bunsen burner flame and pipettes were pulled on the Narashige PP-83 two stage puller. To check that the pipette tip sizes were fairly constant the qualitative bubble test was used (Corey and Stevens, 1983). This test involved connecting the pipette to a 10ml syringe through airtight polythene tubing. With the plunger set on 10mls of air, the pipette tip was placed in a small bottle filled with 70% (v/v) ethanol. Pressure was applied to the plunger until there was the first sign of bubbles emerging from the tip. The reading on the syringe was taken. A value of 6mls was required for the patch pipettes.

The pipettes were then placed on the bubble test syringe again and the tips were dipped into a dimethyldichlorosilane solution (BDH) under positive pressure. The positive pressure prevented blockage of the tips. Coating them in this solution treated the glass in such a way as to make it non-conducting and therefore the noise characteristics were reduced. The coating was allowed to dry at room temperature for at least 1 hour with the pipettes placed in Petri dishes as a protection from dust.

It was necessary to polish the pipette tips since Gigaohm seals can usually only be obtained with smooth pipette tips. This was achieved by melting the tips slightly. To carry this out the pipette was mounted on

a microscope slide and viewed under phase contrast at x250 magnification. The polishing heat was provided by a U-shaped platinum-iridium wire connected to a 6 volt (V) variable output power supply. It was important to have a glass bead melted onto the tip of the wire to prevent metal deposits evaporating onto the pipette tip during the polishing process. The polishing wire was attached to a micromanipulator held on an adjustable clamp to allow it to be positioned and viewed under the microscope. An air jet supplied by a small electrical pump was directed at the tip of the wire to reduce fluctuations in temperature due to drafts of air in the room. Focussing on both the pipette tip and wire under the microscope, the wire was heated causing it to expand. The pipette tip was positioned close to the wire until a slight distortion of the tip was observed which indicated that melting had occurred.

The pipettes were again subjected to the bubble test where a value of 4 to 5mls was required. They were stored in Petri dishes and used within two days of their fabrication.

2.4.2 FILLING PIPETTES

To prevent tip blockages, solutions were passed through a 0.22 μ m (Millipore) filter prior to use. The pipettes were filled in two stages. With a pipette held in a bubble test syringe, the tip was positioned in a small bottle of filtered solution. Negative pressure was applied until the tip could be seen to fill with

solution. The pipette was then back-filled using a syringe and hypodermic needle with a Millipore filter mounting attached. Any bubbles were removed by lightly tapping the pipette.

2.4.3 RECORDING APPARATUS

The patch clamp recordings were made using a List EPC5 patch clamp preamplifier. Unitary currents and patch potentials were monitored on a two channel Nicolet oscilloscope. A voltage pulse was supplied to the patch amplifier from a Grass stimulator so that Gigaohm seal formation could be monitored by measuring pipette tip resistance. All data was recorded on video tape using a video cassette recorder (JVC). The data was converted from analogue to digital signals for video recording by a pulse code modulator (Sony). The filtering on the patch clamp amplifier was set at 3kHz. The recordings were passed through a Neurolog adjustable low pass filter unit which was usually set at 1kHz before monitoring on the oscilloscope. Permanent visual records were obtained by playing the data through a high quality Gould 220 brush chart recorder. The Perspex bath containing the experimental preparation was grounded by an Ag/AgCl wire connected to the ground input of the patch clamp preamplifier headstage.

2.4.4 ARRANGEMENT OF EQUIPMENT

The Perspex bath containing the preparation was exactly the same as that used for intracellular recording. The perfusion system was also essentially the same except that there was only one inflow line from a single reservoir which contained normal Helix saline. No multiwell tap was required.

The equipment was arranged on a baseplate within a copper Faraday cage, essentially in a similar manner as was described for intracellular recordings. Again all pieces of equipment which had the potential to act as aerials for electrical noise were connected to a common ground along with the baseplate and Faraday cage. The high quality signal ground of the patch clamp preamplifier was used. When experiments were being performed, any remaining noise was reduced by bringing down the front of the Faraday cage.

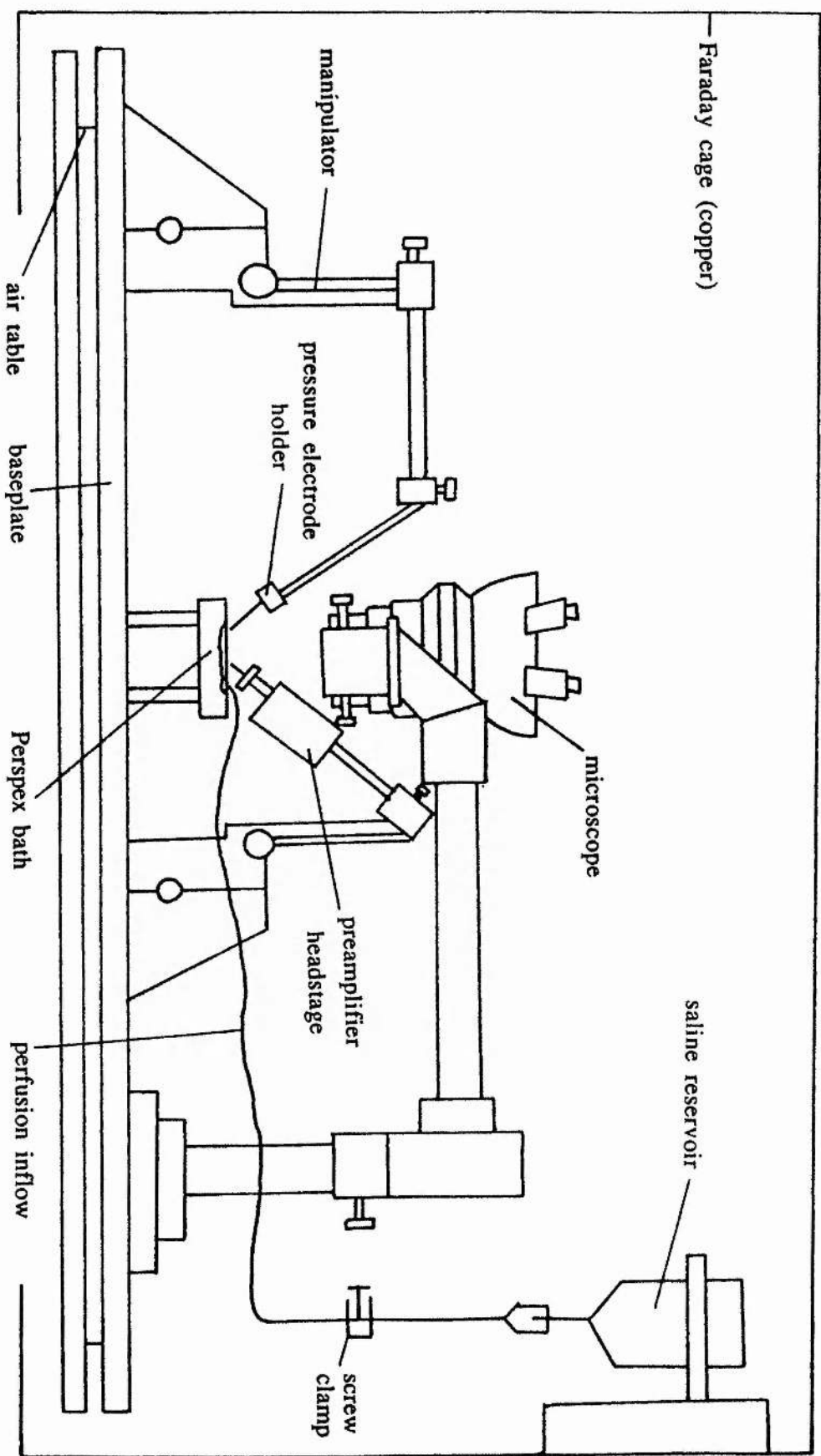
The baseplate was mounted on an anti-vibration air table (Wentworth Laboratories) and air was provided from a high pressure cylinder. A diagram showing the arrangement of equipment is given in Figure 2.5.

2.4.5 FORMATION OF CELL ATTACHED PATCHES AND FMRFamide APPLICATION

Patch pipettes filled with solution were mounted on the Ag/AgCl wire of the preamplifier headstage and held in an airtight pipette holder. Positive or negative pressure could be applied to this holder

Figure 2.5

The arrangement of equipment on the baseplate for patch clamping is shown in this diagram. The steel baseplate and copper Faraday cage were both grounded. As a further protection from electrical noise, the front of the Faraday cage was brought down (not shown) during experiments. The Ag/AgCl grounding wire for the bath is shown connected to the ground input of the patch clamp amplifier headstage. The suction system for removing perfusate from the bath is not shown. Also, the system whereby positive and negative pressure could be applied to the patch pipette holder has been left out. The air table on which the baseplate was mounted is represented.



through a length of plastic tubing attached to a 2ml syringe. Positive pressure was applied prior to placing the pipette in the bath solution to prevent any possible debris blocking the tip. Once the pipette tip had been manoeuvred into the bath saline using a Narashige micromanipulator, the preamplifier was switched on in "search" mode and the direct current offset set to zero. A voltage pulse was passed through the preamplifier to the patch pipette so that tip resistances could be monitored by measuring the resulting current pulses. Pipette tip resistances were typically $2M\Omega$ with normal Helix saline in the pipette.

The pipette tip was carefully moved down towards the cell body of the neurone. When the cell body was touched, the current pulse reduced slightly and the noise level on the current recording was reduced. Gentle suction was then applied from the syringe. If the current pulse reduced to zero with a further noise reduction then this indicated that a high resistance Gigaohm seal had been obtained. On a number of occasions the pipette would rupture the cell membrane, hence moving into a whole cell recording situation. If the patch clamp amplifier was switched to current clamp mode, the resting membrane potential could be measured and the hyperpolarizing effect of FMRFamide monitored. When a cell attached patch had been formed the preamplifier was switched to voltage clamp mode and the pipette potential set as required.

The aim of the experiments on cell attached patches was to apply FMRFamide to the external surface

of the neuronal cell body outwith the patch. FMRFamide was applied by passing controlled pulses of compressed air to a solution contained within an electrode using a Picospritzer unit. The electrodes used were exactly the same as those described for pressure injection during intracellular recording. The ends of FMRFamide containing electrodes were attached to the Picospritzer unit using tight fitting, air tight polythene tubing. They were manoeuvred into position close to the cell body by a hydraulically operated Narashige micromanipulator. Only small pressures and pulse durations were required to deliver sufficient volumes of FMRFamide solution (typically 69kPa, 30ms duration).

2.5 SOLUTIONS

2.5.1 HELIX SALINE

Three salines were used. One was termed "normal saline" and the others " 0Na^+ " and " 0Ca^{2+} ". Constituent listings of each are given below;

Normal saline: 80mM NaCl, 5mM KCl, 5mM MgCl_2 ,
7mM CaCl_2 , 20mM Hepes, pH 7.5 with NaOH.

0Na^+ saline: 160mM sucrose, 5mM KCl, 5mM MgCl_2 ,
7mM CaCl_2 , 20mM Hepes, pH 7.5 with KOH.

0Ca^{2+} saline: 80mM NaCl, 5mM KCl, 9.5mM MgCl_2 ,
2mM EGTA, 20mM Hepes, pH 7.5 with NaOH.

EGTA and Hepes buffer (free acid) were obtained from Sigma. All other salts were obtained from BDH.

2.5.2 NEUROPEPTIDE SOLUTIONS

A description for the preparation of the 5mM FMRFamide solution used for iontophoresis has been described in the iontophoresis section of intracellular recording (2.3.4).

For pressure ejection during patch clamping experiments, FMRFamide was dissolved in normal saline at a concentration of 100 μ M and filtered through a 0.22 μ m Millipore filter. FMRFamide was obtained from Peninsula Laboratories.

2.5.3 MEMBRANE SOLUBLE SECOND MESSENGER ANALOGUES, ENZYME ACTIVATORS AND INHIBITORS

8-(-4) CPT cyclic AMP (Boehringer Mannheim), dibutyryl cyclic AMP (Sigma), dibutyryl cyclic GMP (Sigma), 8-bromo cyclic GMP (Sigma) and IBMX (Sigma) were dissolved directly in normal Helix saline and used at concentrations of 100 μ M and 1mM.

Concentrated stock solutions of phorbol 12,13-dibutyrate (Sigma) and arachidonic acid (Sigma) were initially dissolved in 100% DMSO (Sigma) at concentrations of 100mM. Aliquots were diluted down to 50 μ M phorbol 12,13-dibutyrate (0.05% (v/v) DMSO) and 50 μ M arachidonic acid (0.05% (v/v) DMSO) in normal saline. Okadaic acid was received (Prof. P. Cohen,

University of Dundee) at a concentration of 10mM in 100% DMSO. Aliquots were diluted down to 1 μ M in normal saline (0.01% (v/v) DMSO).

2.5.4 DRUGS TO CHARACTERIZE THE FAST Na⁺ RESPONSE

CoCl₂ (BDH), tetrodotoxin (Sigma) and amiloride (Sigma) were all dissolved in normal saline at concentrations of 1mM, 50 μ M, and 100 μ M respectively. Lignocaine (Sigma) was firstly dissolved in 100mM HCl to make the lignocaine hydrochloride salt. This was then diluted in normal saline and the pH was adjusted to 7.5 with NaOH. The final concentration of lignocaine was 1mM.

2.5.5 INTRACELLULARLY INJECTED COMPOUNDS

a) GTP γ S (Sigma), EGTA and inositol 1,4,5-trisphosphate (IP₃).

GTP γ S (Sigma), EGTA (Sigma) and IP₃ (Amersham) were diluted to concentrations of 10mM, 700mM and 0.78mM respectively in a solution which consisted of 100mM KCl, 20mM Hepes, 1% (w/v) fast green dye, pH 7.4 with KOH. The solvent was passed through a 0.22 μ m Millipore filter.

b) Pertussis toxin.

Two solutions were required to activate pertussis toxin. The constituents of the solutions are listed below.

Solution 1 : 10mM DTT (Sigma), 25mM MOPS buffer,
200mM KCl, pH 7.5 with KOH.

The solution was made up in distilled water.

Solution 2 : 10mM NAD (Sigma), 10mM ATP (Sigma) made
up in an aliquot of solution 1.

1% (w/v) fast green dye was added to solution 2. The solution was then passed through a 0.22 μ m Millipore filter. Pertussis toxin (Sigma) was dissolved in Solution 2 to the required concentration of 0.5mg/ml and the temperature of the reaction mixture was raised to 35°C for 15 minutes in a water bath. This allowed DTT to reduce disulphide bonds and activate the toxin.

Chapter 3

Results

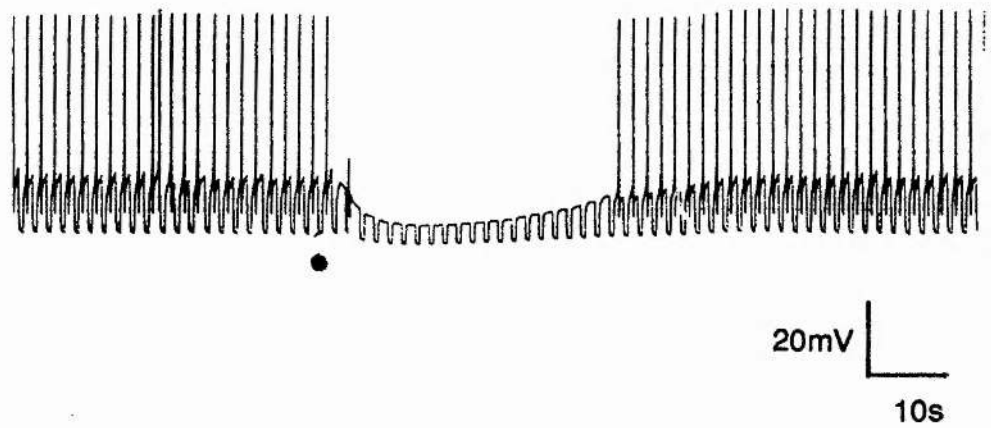
3.1 THE SLOW HYPERPOLARIZING FMRFamide RESPONSE OF THE C1 NEURONE

FMRFamide application to this neurone resulted in a slow hyperpolarization at a resting membrane potential which averaged -50mV (40 neurones). The hyperpolarization was associated with a decrease in input resistance as indicated by the reduction in size of the voltage deflections elicited by constant hyperpolarizing current pulses. Although the current clamp record in Figure 3.1a shows the C1 neurone spiking at the resting membrane potential, this did not normally occur. The spikes appeared here as a rebound artifact caused by the delivery of hyperpolarizing current pulses.

When the C1 neurone was voltage clamped at -45mV , the membrane current underlying the hyperpolarization was clearly seen. The current was slow and outward in direction. The average time taken between FMRFamide application and the maximum amplitude of the response was approximately 7 seconds. The decrease in input resistance caused by FMRFamide was observed as an increase in amplitude of the current pulses which were elicited by a constant hyperpolarizing voltage step.

The relation between the current response and the holding membrane potential is shown in Figure 3.2. The reversal potential for the response was approximately -70mV . The overall relationship was not linear. The nature of the relationship was characteristic of the constant field equation which describes the movement of

a) $V_m -48\text{mV}$



b) $V_h -45\text{mV}$

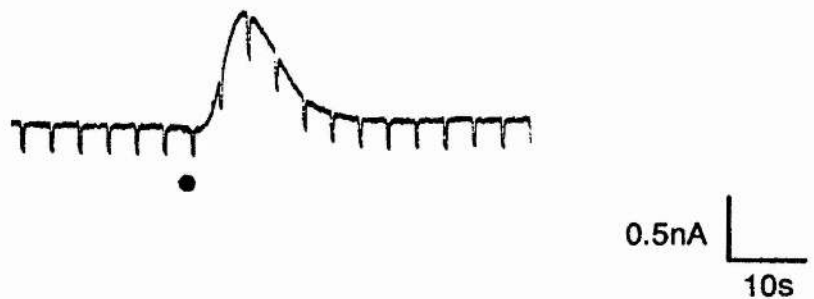


Figure 3.1 a) and b)

Recordings of the slow, outward current induced by FMRFamide in two different C1 neurones, one under current clamp and the other under voltage clamp conditions. a) Current clamp recording from a neurone which had a resting membrane potential (V_m) of -48mV . Voltage deflections resulting from constant hyperpolarizing current pulses were used to monitor input resistance. b) Voltage clamp recording from another neurone at a holding potential (V_h) of -45mV . Current deflections induced by a constant hyperpolarizing voltage step were used to monitor input resistance. FMRFamide was applied in both records at the points marked by the black dots.

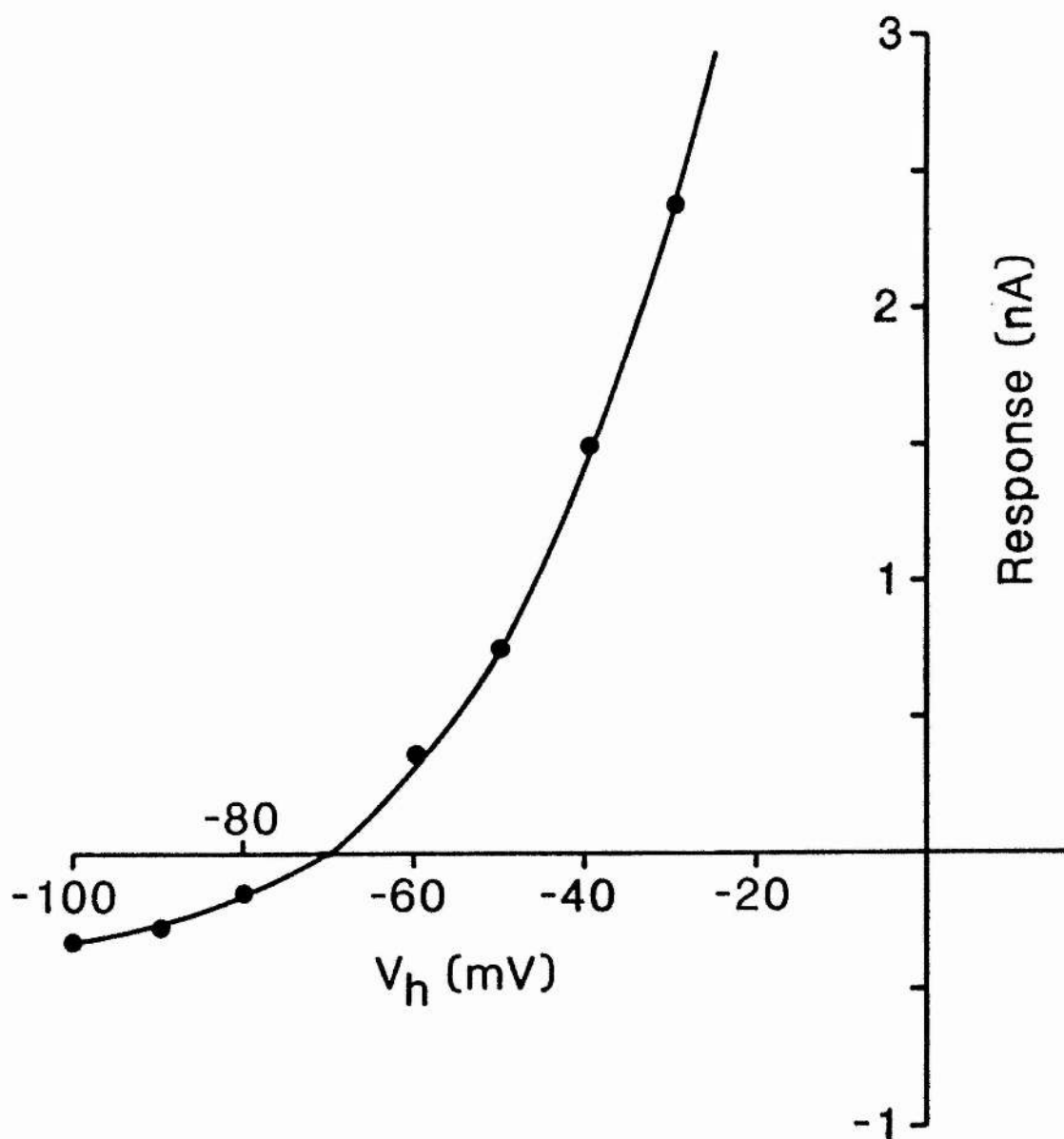


Figure 3.2

The relationship between amplitude of the FMRamide response and holding potential. The reversal potential was -70mV.

an individual ionic species moving across the membrane by simple diffusion along a potential gradient. The value of the reversal potential indicated that the outward current response to FMRFamide may have been due to the movement of K^+ . Previous experiments with ion substitution and ion channel blockers showed that the outward current was indeed carried by K^+ (Cottrell, 1982; Cottrell et al, 1984).

Cottrell (1982) also showed that when the neurone was depolarized from resting membrane potential, the response became biphasic with an inward, as well as an outward current component. Further depolarization changed the response to an inward current only. The inward current was subsequently studied and found to be the result of the closure of K^+ channels (Cottrell et al, 1984). This voltage dependent inward current is similar to that induced by 5-HT in the C1 neurone at depolarized potentials (Cottrell, 1982).

3.2 ANALYSIS OF THE INTRACELLULAR MECHANISM WHICH UNDERLIES THE FMRFamide INDUCED K^+ CURRENT IN THE C1 NEURONE

The slow nature of the FMRFamide response suggests that the receptor may be linked to the ion channel through the activation of a guanine nucleotide binding protein (G protein) and possibly through a second messenger pathway. Many, but not all of the actions of these second messenger cascades are mediated by protein phosphorylation. A number of second messenger cascades

have now been characterized, examples of which involve cyclic AMP and cyclic GMP-mediated protein phosphorylation, the breakdown products of phosphatidylinositol 4,5 bisphosphate (IP_3 and diacylglycerol), arachidonic acid and its metabolites and finally, intracellular Ca^{2+} . All of the above are candidates for the mechanism which underlies the FMRFamide response under investigation. Each has been tested and is referred to in the following sections.

3.3 INVOLVEMENT OF A G PROTEIN

G proteins have been identified in almost all cells in every tissue and their major role is known to be the transduction of various extracellular signals by interacting between receptor molecules and effector enzymes or ion channels. Several different molecular probes that regulate G protein activity are now available to study the modulation of ion channels. The substances used in the following experiments were GTP γ S and pertussis toxin (islet activating protein).

3.3.1 GTP γ S INJECTION EXPERIMENTS

GTP γ S is a non-hydrolysable analogue of GTP which has been used to cause persistent activation of G proteins (Rodbell, 1980). GTP γ S is not specific for the activation of any one type of G protein. For example, it can activate G proteins which stimulate adenylate

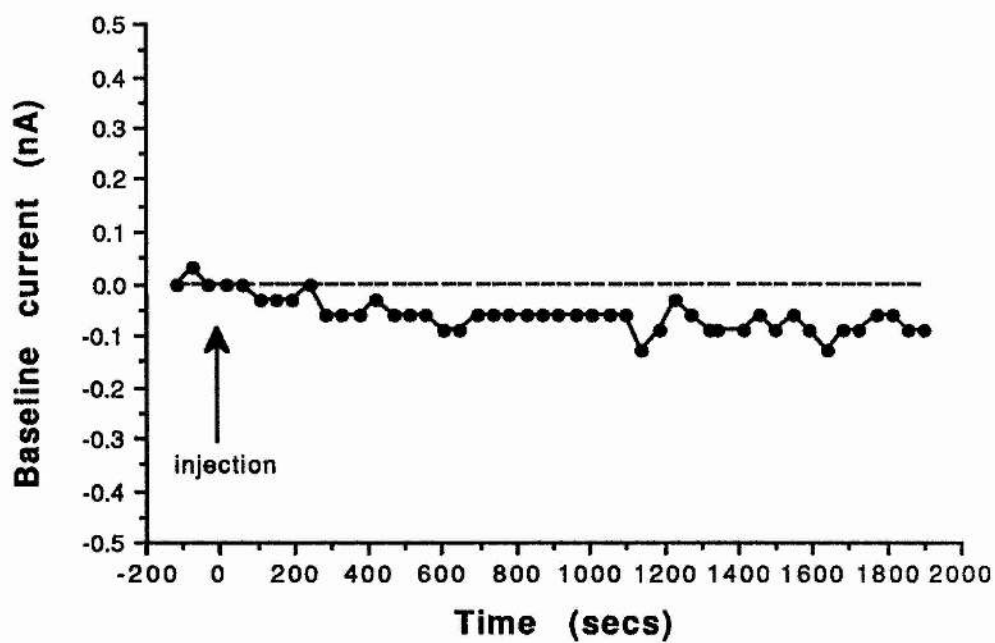
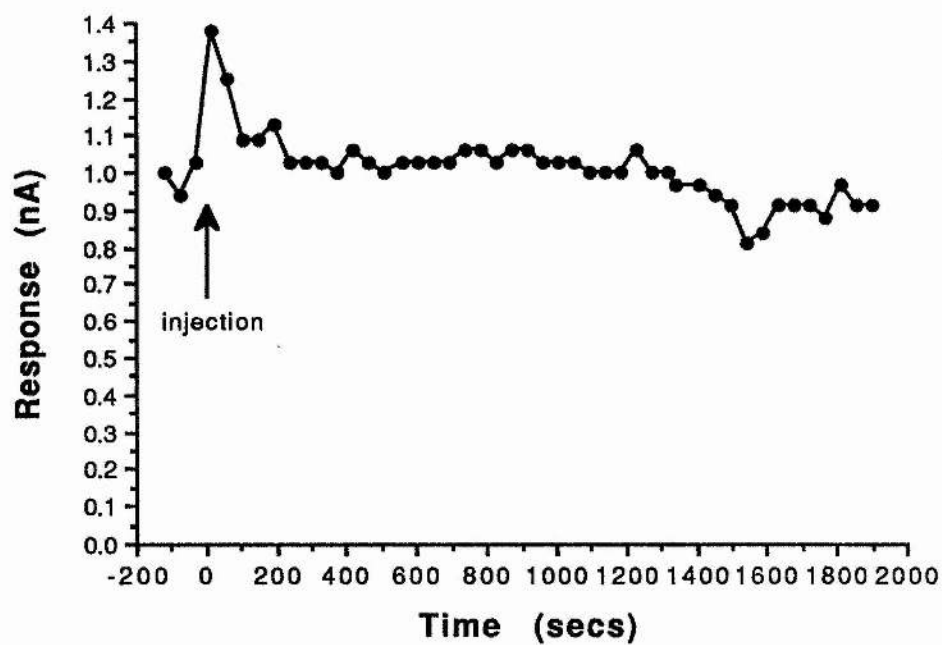
cyclase (G_s), G proteins which inhibit adenylate cyclase (G_i) and other G proteins (G_o).

GTP γ S was dissolved in a solvent solution consisting of 100mM KCl, 20mM Hepes buffer, 1% (w/v) fast green, pH 7.4 with KOH (Brezina, 1988). Injection of the solvent alone (3 replicate experiments) had no long-term effect on the amplitude of the FMRFamide response but it did induce a very slight inward current (Figure 3.3). However, the inward current was small and did not effect the experiments with GTP γ S. Figure 3.4 shows the record of an experiment where GTP γ S was injected at a concentration of 10mM. Taking the intracellular fluid volume and volume of GTP γ S injected into account (see Methods), the estimated final intracellular concentration of GTP γ S was 10-100 μ M. This concentration range was similar to that used by Sasaki and coworkers, (1987a and b) when injecting GTP γ S into Aplysia neurones. GTP γ S induced an outward current which was associated with an increase in membrane conductance, as indicated by the increase in size of the current deflections. Also, the outward current responses to FMRFamide were depressed following the development of the outward GTP γ S current. The final level of the baseline membrane current was approximately equal to that of the peak outward current of the control responses to the neuropeptide.

If the record in Figure 3.4 is examined closely, it appears that the baseline membrane current was already moving slightly outward with the GTP γ S electrode impaled into the neurone prior to injection.

Figure 3.3

The effect of injection of the solvent used for the GTP γ S experiments into the cell body of a C1 neurone. The neurone was voltage clamped at -45mV. The upper graph shows that solvent injection transiently increased the amplitude of the FMRFamide response. The response amplitude then returned close to control levels. The solvent injection also produced a very small inward current which persisted for the duration of the experiment (lower graph). The dashed line on the lower graph indicates the baseline current level at the time of injection (time zero). The point of injection is marked by the vertical arrow on each graph.



V_h -45mV

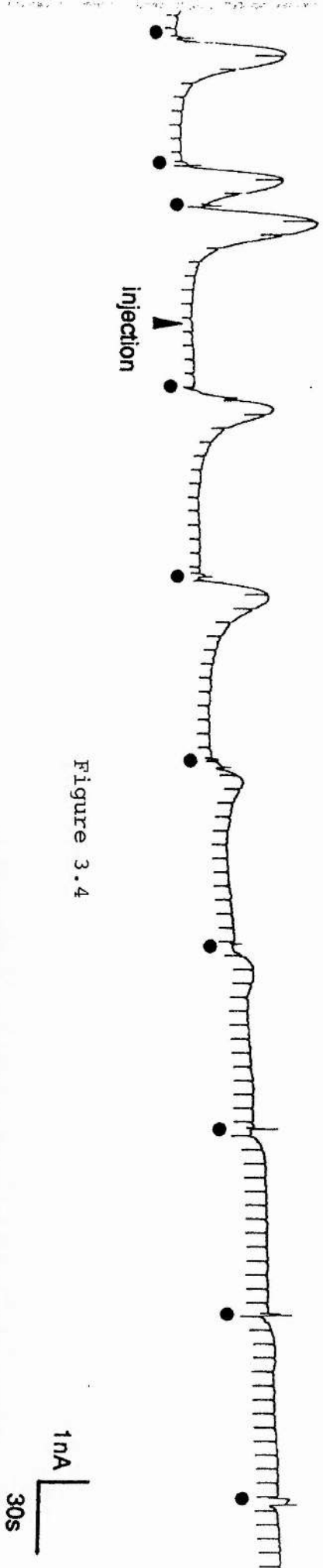


Figure 3.4

The influence of injecting 10mM GTPs on the amplitude of the FMRFamide response and baseline current level recorded under voltage clamp conditions at V_h -45mV. FMRFamide was applied at the points marked with the black dots and GTPs was injected at the arrow head. Note the slowly developing baseline outward current. This was accompanied by a gradual increase in membrane conductance, indicated by the increase in size of the current pulses evoked by a constant hyperpolarizing voltage step. The baseline outward current occluded the FMRFamide responses.

In some neurones this outward current was larger and developed only 1-2 minutes after impaling the neurone. With these neurones it was difficult to elicit any FMRamide responses. These events indicated that GTP γ S was leaking into the neurones prior to injection.

These results strongly suggest the involvement of a G protein in the FMRamide induced, outward K⁺ current.

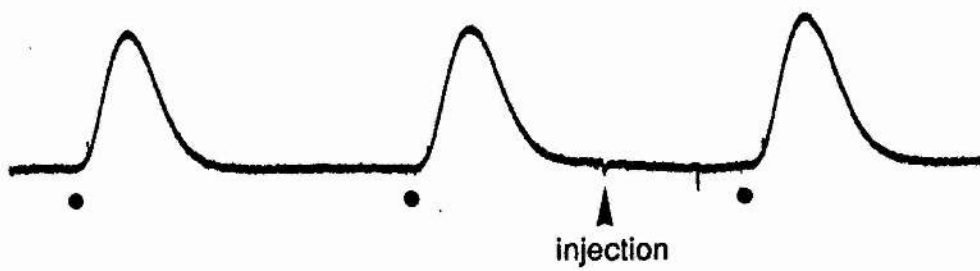
3.3.2 PERTUSSIS TOXIN EXPERIMENTS

The bacterial toxin, pertussis toxin, inhibits the actions of the G proteins G_i (inhibits adenylate cyclase) and G_o (some other function) in the presence of nicotinamide adenine dinucleotide (NAD) (Neer *et al*, 1984) by ADP-ribosylation of the α subunits of these G proteins. Figure 3.5 shows a voltage clamp recording where pre-activated pertussis toxin was injected at a concentration of 0.5mg/ml to give an estimated intracellular concentration of 0.5-5 μ g/ml (see Methods). Pertussis toxin quite clearly reduced the amplitude of the FMRamide response (to 42% of its original amplitude) over a period of approximately 1200 seconds (20 minutes). Injection of the activation solution (200mM KCl, 10mM DTT, 10mM NAD, 25mM MOPS, 1% (w/v) fast green, pH 7.4) had no effect on the amplitude of the FMRamide responses. The results obtained with the activation solution (control) and pertussis toxin are compared graphically in Figure 3.6.

Figure 3.5

The effect of pre-activated pertussis toxin injection into the cell body of a C1 neurone voltage clamped at -45mV . FMRFamide was applied at the points marked by the black dots and pertussis toxin (0.5mg/ml) was injected at the point indicated by the arrowhead. The amplitude of the FMRFamide responses gradually reduced over a period of approximately 1200 seconds (20 minutes) after injection. The time marked above each record refers to the first FMRFamide response in that record relative to pertussis toxin injection (time zero).

-71s



379s



1144s



V_h -45mV

0.5 nA
10s

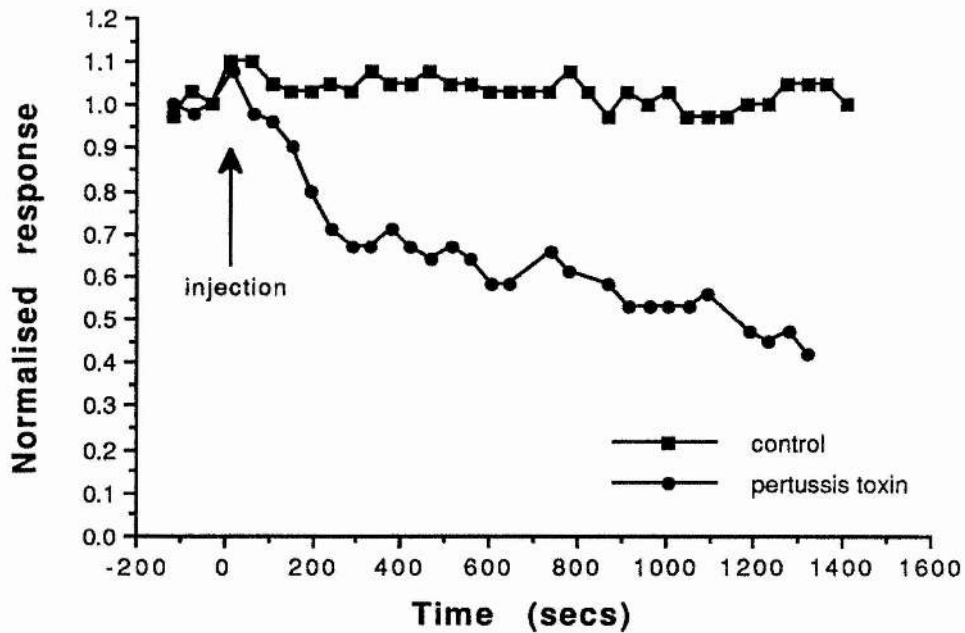


Figure 3.6

The comparison of the effects of injecting activating solution (control) and activating solution containing pertussis toxin on the amplitude of the FMRFamide response of C1 neurones voltage clamped at -45mV . The FMRFamide responses were normalised. The point of injection for both experiments is shown by the vertical arrow. The activating solution had no effect but pertussis toxin reduced the amplitude of the responses.

Similar effects to those described above were observed in three other C1 neurones.

3.4 CYCLIC NUCLEOTIDES

Of the several second messengers which have been characterized and shown to be the link between neurotransmitter receptors and ion channels, the two most studied have been the cyclic nucleotides, cyclic AMP and cyclic GMP.

Intracellular levels of the cyclic nucleotides can be increased experimentally either by direct injection or by exposing the neurones to membrane soluble analogues. The use of membrane soluble analogues is technically simpler. This method has been used in these studies. Usually very high concentrations ($100\mu\text{M}$ - 1mM) of the membrane soluble analogues must be applied before any effects can be detected. There are two reasons for this. Firstly, neurones may not be very permeable to a particular cyclic nucleotide analogue and secondly, such analogues may be subject to rapid metabolism by phosphodiesterase enzymes. The levels of cyclic nucleotides in a cell are normally controlled by the synthetic and degradative processes. The inhibition of phosphodiesterase enzymes therefore, can also be used to elevate the levels of cyclic nucleotides in cells.

3.4.1 THE CYCLIC AMP SYSTEM

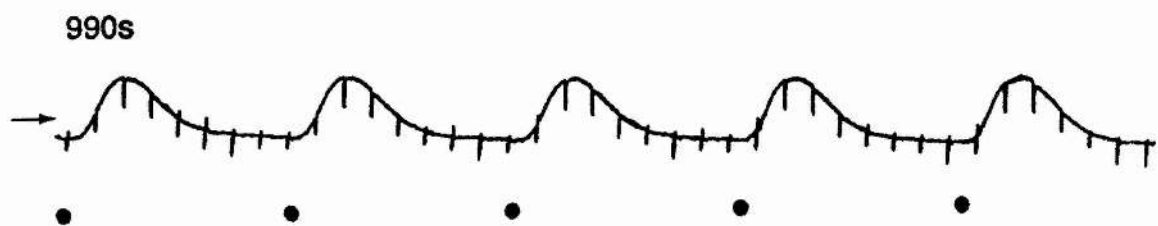
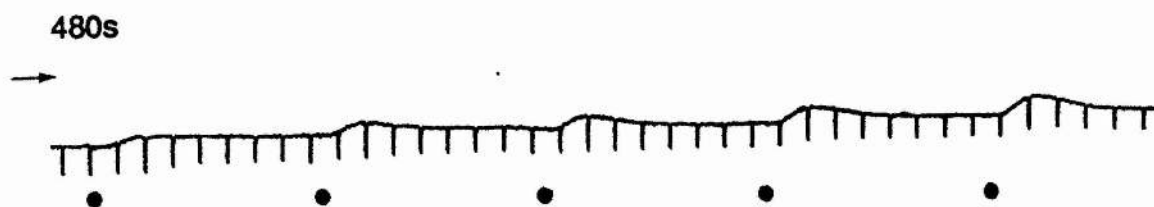
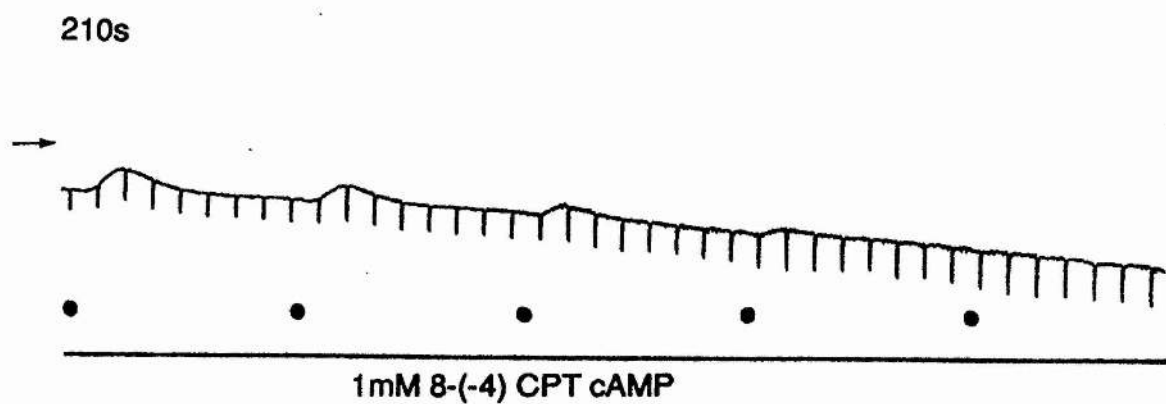
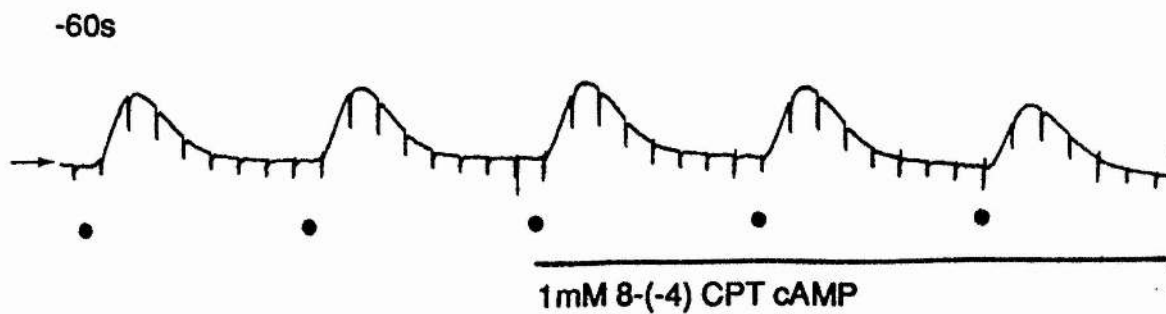
The two membrane soluble analogues of cyclic AMP used in these experiments were 8-(-4) CTP cyclic AMP and dibutyryl cyclic AMP. The 8-(-4) CPT cyclic AMP derivative has been reported to be a hundred times more effective than dibutyryl cyclic AMP in the activation of cyclic AMP dependent protein kinase A in rat liver and to be more resistant to phosphodiesterase activity (Miller *et al*, 1975).

The voltage clamp recordings in Figure 3.7 illustrates the actions of 1mM 8-(-4) CPT cyclic AMP when applied to a C1 neurone. This substance had two clear effects. Firstly, the amplitude of the FMRFamide response was rapidly reduced (within 60 seconds) leading to complete abolition of the response after approximately 300 seconds (5 minutes) (2 replicate experiments (n=2)). Secondly, an inward current was induced which reached a maximum amplitude in 360 seconds (7 minutes) (n=2). This inward current was associated with an increase in size of the current pulses elicited by a constant voltage step, indicating that the membrane conductance had increased to some ion(s). Both effects were reversed on washing for 720 seconds (12 minutes) with normal saline. No further investigations were made on the inward current. Data from the experiment are shown in Figure 3.8.

In another experiment 1mM 8-(-4) CPT cyclic AMP also reduced the FMRFamide response but only to 30% of its original amplitude and the inward current was also

Figure 3.7

The effect of 1mM 8-(-4) CPT cyclic AMP dissolved in the perfusing saline on the amplitude of the FMRFamide response and baseline current level for a C1 neurone voltage clamped at -45mV. Application of 8-(-4) CPT cyclic AMP is indicated by the lines underneath the records. The time, relative to the start of application (time zero) is marked above each section of record. The arrows to the left of each record show the original level of the baseline current at time zero. Current deflections evoked by a constant hyperpolarizing voltage step were used to monitor input resistance. Note the reversible inward current induced by 8-(-4) CPT cyclic AMP, the decrease in input resistance and the reversible reduction of the amplitude of the FMRFamide response. FMRFamide was applied at the points marked by the black dots.

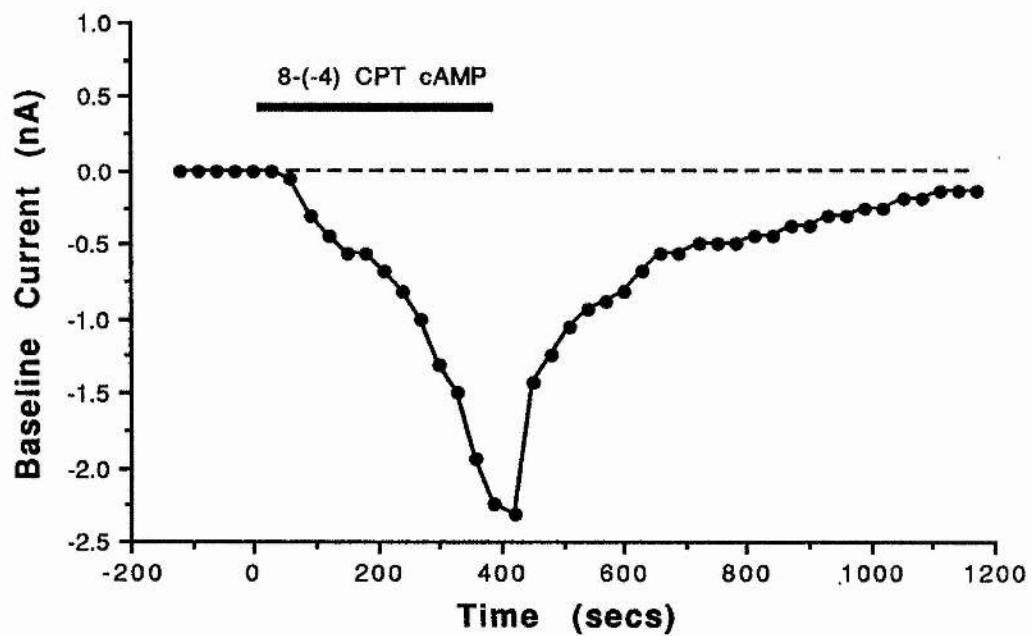
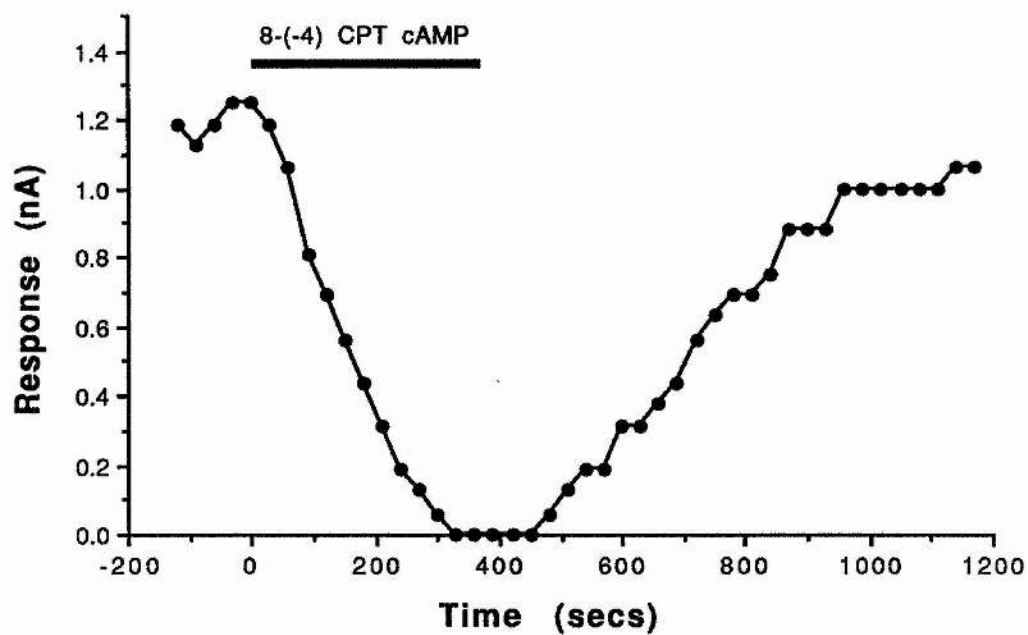


$V_h -45\text{mV}$

1nA
10s

Figure 3.8

The influence of 1mM 8-(-4) CPT cyclic AMP on a C1 neurone which was voltage clamped at -45mV. The upper graph shows the reversible reduction of the amplitude of the FMRFamide response and the lower graph the reversible baseline inward current. The application of 8-(-4) CPT cyclic AMP is marked by the bars on each graph. The original baseline current level at time zero is indicated by the dashed line on the lower graph.



smaller. This was likely to have been due to a poorer penetration of the 8-(-4) CPT cyclic AMP.

When dibutyryl cyclic AMP was applied to the C1 neurone it had absolutely no effect on either the FMRFamide response amplitude or the baseline membrane current ($n=3$), possibly because the dibutyryl derivative is more susceptible to phosphodiesterases and/or is less potent on the activation of cyclic AMP dependent protein kinase A (see above).

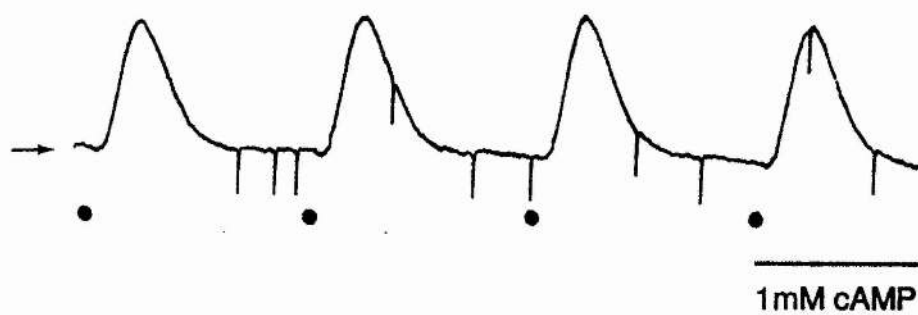
Cyclic AMP is relatively membrane insoluble and was used as a control to check whether 8-(-4) CPT cyclic AMP could have a non-specific action on the external surface of the membrane. An example recording of such an experiment is shown in Figure 3.9. External application of 1mM cyclic AMP for a period of up to 690 seconds (11.5 minutes) had no effect on the baseline membrane current level or the amplitude of the FMRFamide response ($n=2$). The data obtained from the experiment are plotted in Figure 3.10. This result indicates that the effects of 8-(-4) CPT cyclic AMP were unlikely to have been due to an external, non-specific action.

To implicate a substance as a second messenger, application of the second messenger should in some way mimic the action of the ligand in question. Since 8-(-4) CPT cyclic AMP did not cause an outward current to develop or potentiate the FMRFamide response, cyclic AMP can be ruled out as the second messenger for the FMRFamide response.

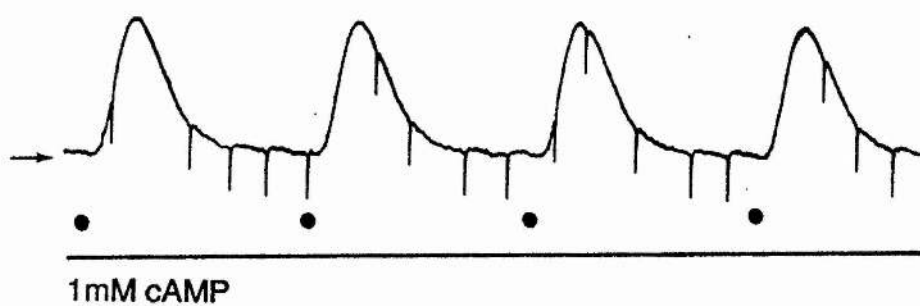
Figure 3.9

This control experiment shows that extracellularly applied 1mM cyclic AMP in the perfusing saline had no effect on the amplitude of the FMRFamide response or baseline current level of a C1 neurone voltage clamped at -45mV. Cyclic AMP application is marked by the lines underneath the records. The time indicated above each record refers to the first FMRFamide response in that record relative to the start of application (time zero). The arrows to the left of the records show what was the baseline current level at time zero. FMRFamide was pulsed onto the neurone at points marked by the black dots. The inward deflections on the records are unclamped axon spikes.

-90s



600s

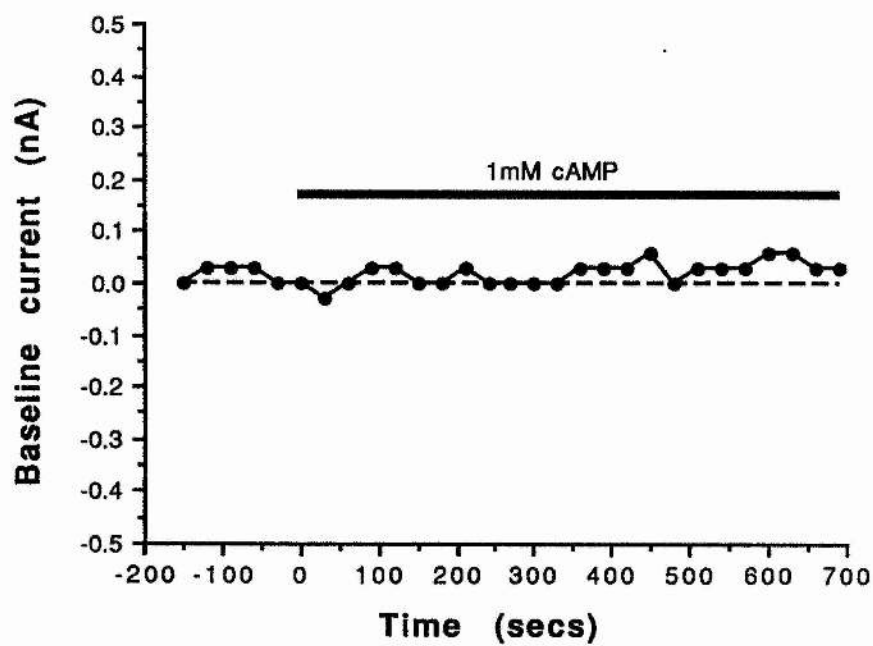
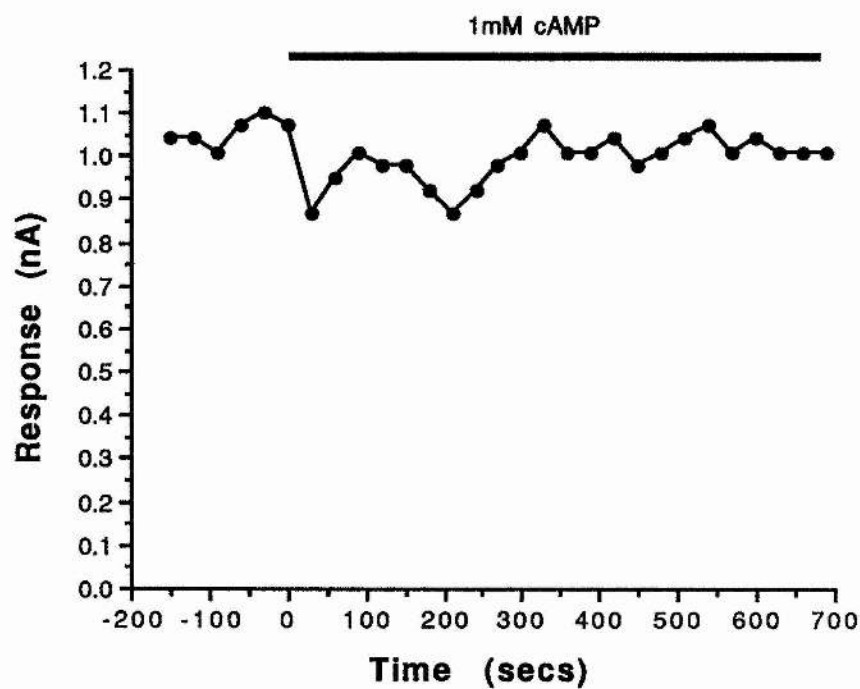


$V_h -45\text{mV}$

0.5nA
10s

Figure 3.10

Data obtained from an experiment where 1mM cyclic AMP was applied to a voltage clamped C1 neurone held at -45mV, showing the lack of effect on the amplitude of the FMRFamide response (upper graph) and baseline current level (lower graph). Cyclic AMP application is marked by the bars on each graph. The original level of the baseline current at time zero is shown by the dashed line on the lower graph.



3.4.2 THE CYCLIC GMP SYSTEM

The membrane soluble analogues of cyclic GMP used in these experiments were 8-bromo cyclic GMP and dibutyryl cyclic GMP. Exposure to 1mM 8-bromo cyclic GMP for 910 seconds (approximately 15 minutes) had no effect on the amplitude of the FMRFamide response but it did induce a relatively large inward current (2.0nA) over this time period (Figure 3.11). The inward current induced by 8-bromo cyclic GMP had not reached a plateau value by the end of the experiment (n=3). This inward current was not investigated further.

With 1mM dibutyryl cyclic GMP application there was no change in either the amplitude of the FMRFamide response or the baseline membrane current (n=2). This was similar to the situation with dibutyryl cyclic AMP.

These results suggest that cyclic GMP is not involved in the outward current evoked by FMRFamide in the C1 neurone.

3.4.3 PHOSPHODIESTERASE INHIBITION

Inhibition of the phosphodiesterase enzymes which metabolize cyclic AMP and cyclic GMP is another method whereby the intracellular concentrations of these cyclic nucleotides can be increased. The membrane soluble xanthine compound IBMX was the phosphodiesterase enzyme inhibitor used in this study.

Figure 3.12 shows the influence of 1mM IBMX on the FMRFamide response and baseline current of a C1 neurone

Figure 3.11

Data from an experiment where 1mM 8-bromo cyclic GMP was applied to a C1 neurone voltage clamped at -45mV. It had no effect on the amplitude of the FMRFamide response (upper graph) but did induce an inward current which had not reached a plateau after 930 seconds (15.5 minutes) (lower graph). 8-bromo cyclic GMP application is shown by the bars on each graph. The baseline current level at time zero is marked by the dashed line on the lower graph.

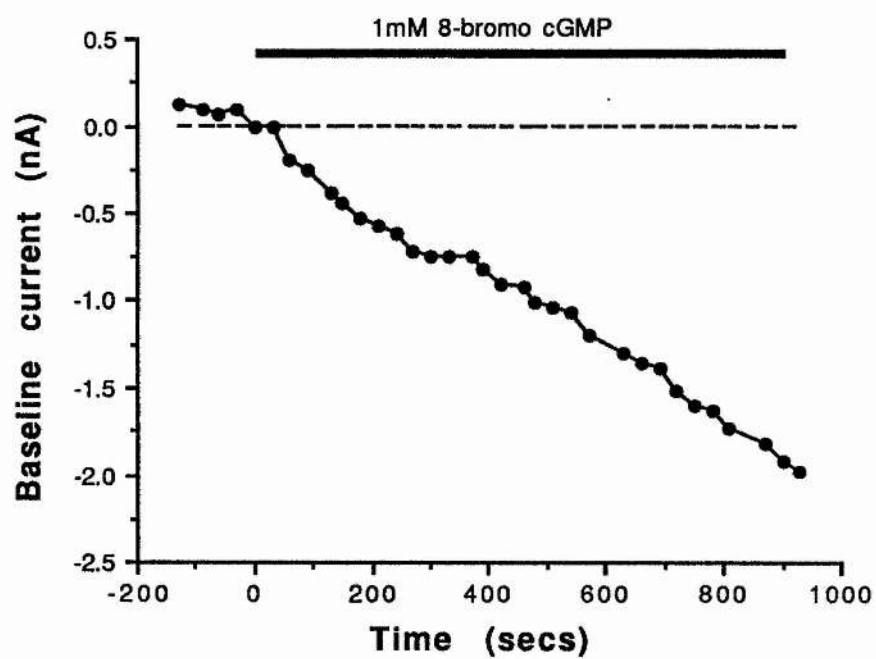
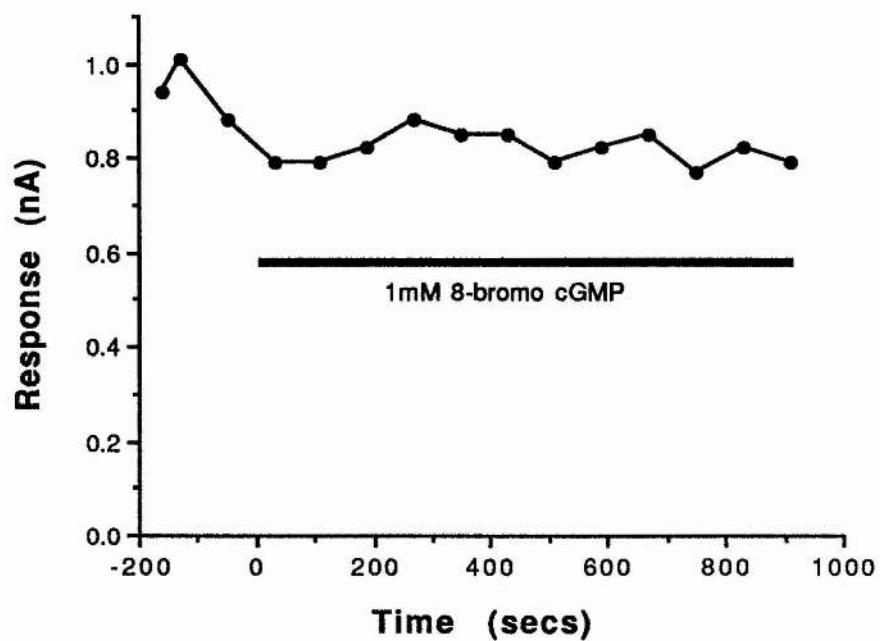
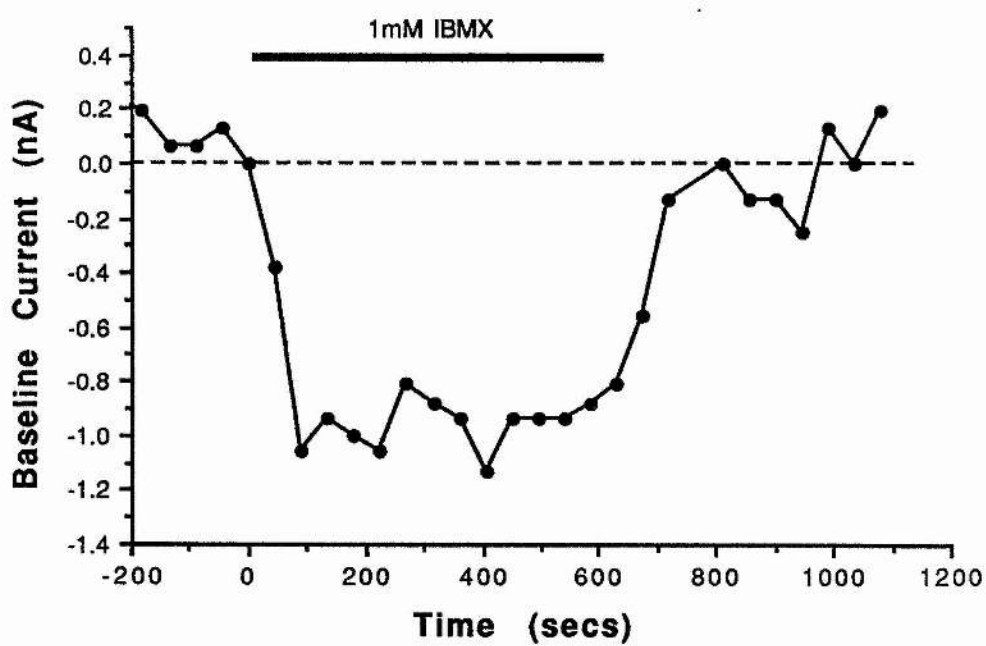
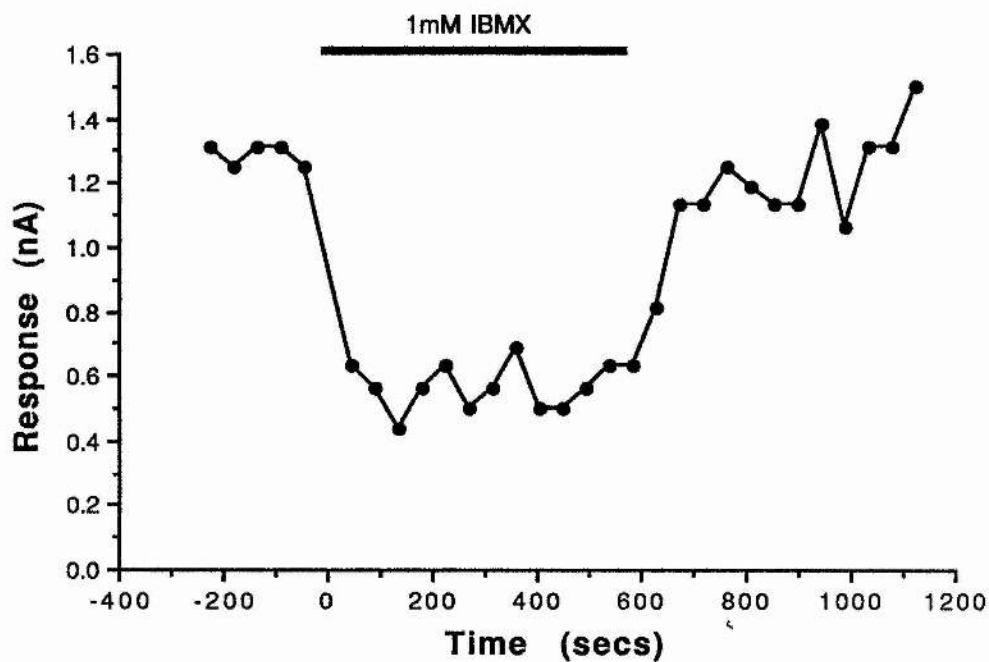


Figure 3.12

Data obtained when examining the influence of 1mM IBMX application on the amplitude of the FMRFamide response and baseline current level of a C1 neurone voltage clamped at -45mV. IBMX reversibly reduced the amplitude of the FMRFamide response (upper graph) and induced a reversible inward current (lower graph). Application of IBMX is indicated by the bars on each graph. The original baseline current level at time zero is shown by the dashed line on the lower graph.



which was voltage clamped at -45mV . The reduction in response amplitude reached a maximum (approximately 35% of the original response) in 120 seconds (2 minutes). The response was completely reversed after 480 seconds (8 minutes) of washing with normal saline ($n=3$). IBMX also induced an inward current (Figure 3.12 lower graph) which reached a maximum amplitude in 120 seconds (2 minutes). This effect was reversed after 420 seconds (7 minutes) of washing ($n=3$). These results parallel those seen when 8-(-4) CPT cyclic AMP was applied to the C1 neurone.

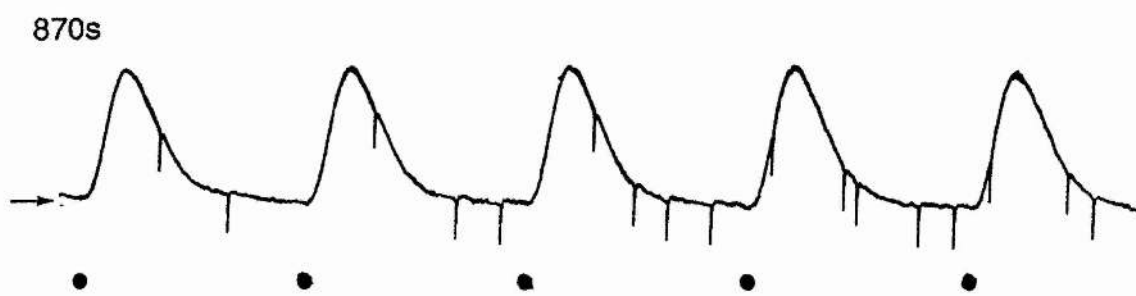
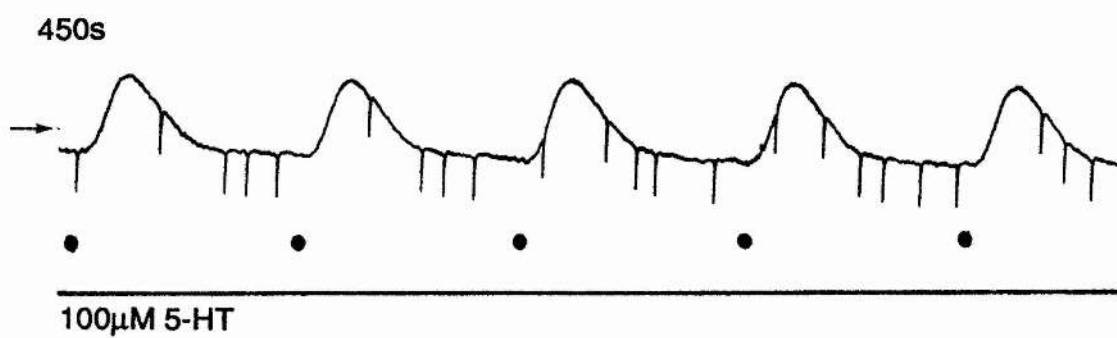
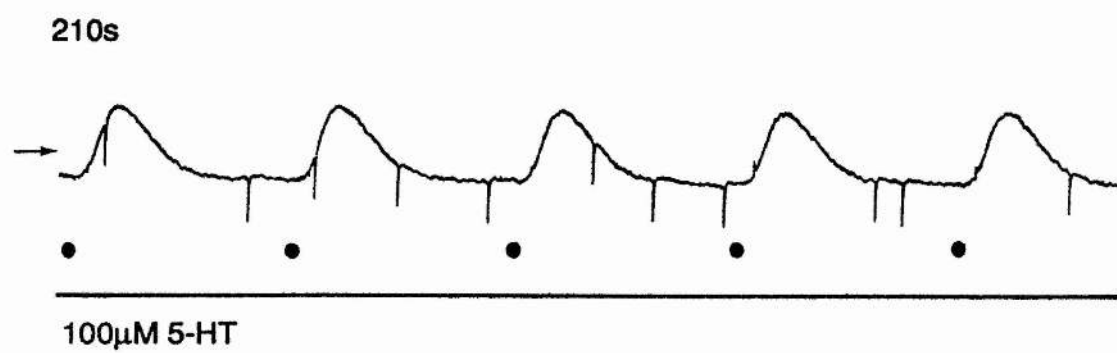
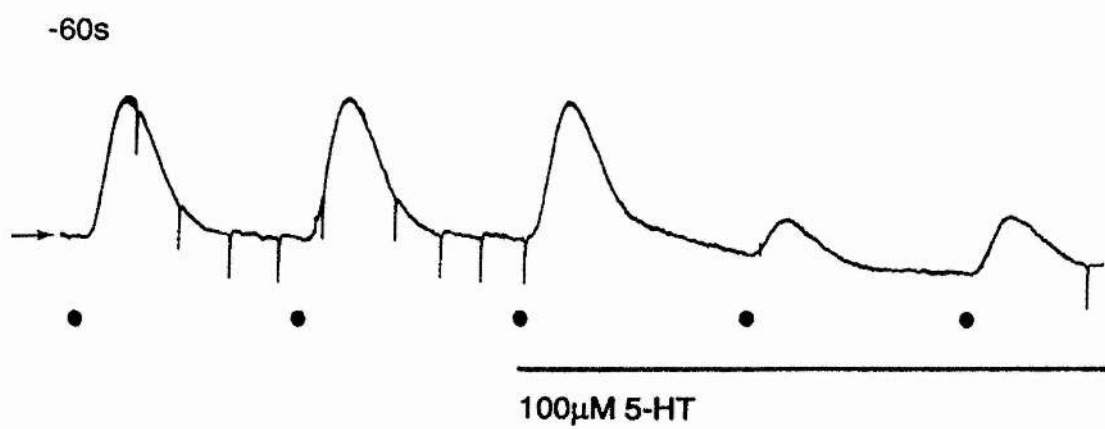
3.5 A ROLE FOR 5-HYDROXYTRYPTAMINE (5-HT)

The reduced amplitude of the FMRFamide response obtained in the presence of elevated cyclic AMP levels closely resembled observations made by Brezina and co-workers, (1987a) with Aplysia neurones. They discovered that 5-HT could mimic the effect of cyclic AMP and therefore suggested that the suppression of the FMRFamide induced outward current by 5-HT was mediated by an increase in intracellular cyclic AMP levels.

Figure 3.13 shows the effect of $100\mu\text{M}$ 5-HT on FMRFamide responses in a C1 neurone. 5-HT reduced the FMRFamide response to between 20 and 40% of its original amplitude within 30 seconds of application. 5-HT also induced a small inward current (0.2nA). A full recovery of the FMRFamide response and the inward current occurred with washing the preparation with

Figure 3.13

The effect of $100\mu\text{M}$ 5-HT application on the amplitude of the FMRFamide response and baseline current level on a C1 neurone, voltage clamped at -45mV . 5-HT reversibly reduced the amplitude of the FMRFamide response and induced an inward current. The reduction in the amplitude of the FMRFamide response reduced in the continued presence of 5-HT. 5-HT application is shown by the lines beneath the records. The time marked above each record refers to the first FMRFamide response in that record relative to the start of 5-HT application which was time zero. The baseline current level at time zero is shown by the arrows to the left of the records. Pulses of FMRFamide were administered to the neurone at points indicated by the black dots. The inward deflections on the records are unclamped axon spikes.



V_h -45mV

0.5nA
10s

normal saline (n=6). The data are plotted in Figure 3.14.

5-HT was also applied to the C1 neurone in the presence of 1mM IBMX so that levels of cyclic AMP were elevated. The data obtained are plotted in Figure 3.15. The data generated with 5-HT alone are also plotted in Figure 3.15 for comparison. Two obvious differences in these data can be seen as regards both the FMRFamide response and the 5-HT induced inward current (n=2). Firstly, the reduction of the FMRFamide response was greater with the inclusion of IBMX, reaching to only 7% of the original response amplitude remaining. The 5-HT induced inward current was much larger, reaching a maximum amplitude of 1.2nA. Secondly, upon washing with normal saline the recovery times were longer. In fact the FMRFamide response did not fully recover for the duration of the experiment.

These results indicated that 5-HT may be a transmitter substance that normally induces elevation of cyclic AMP, which in turn causes a reduction in amplitude of the FMRFamide response. Also, the 5-HT induced inward current is probably mediated by an increase in cyclic AMP levels.

3.6 INOSITOL 1,4,5-TRISPHOSPHATE (IP₃) AND PROTEIN KINASE C

Receptor-mediated hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP₂) by phospholipase C, is another mechanism for information

Figure 3.14

Data from the experiment where $100\mu\text{M}$ 5-HT was applied to a C1 neurone voltage clamped at -45mV . 5-HT reversibly reduced the amplitude of the FMRFamide response (upper graph) and produced a small, reversible inward current (lower graph). The reduction in the amplitude of the FMRFamide response reduced in the continued presence of 5-HT. 5-HT application is indicated by the bar in each graph. The baseline current level is indicated by the dashed line on the lower graph.

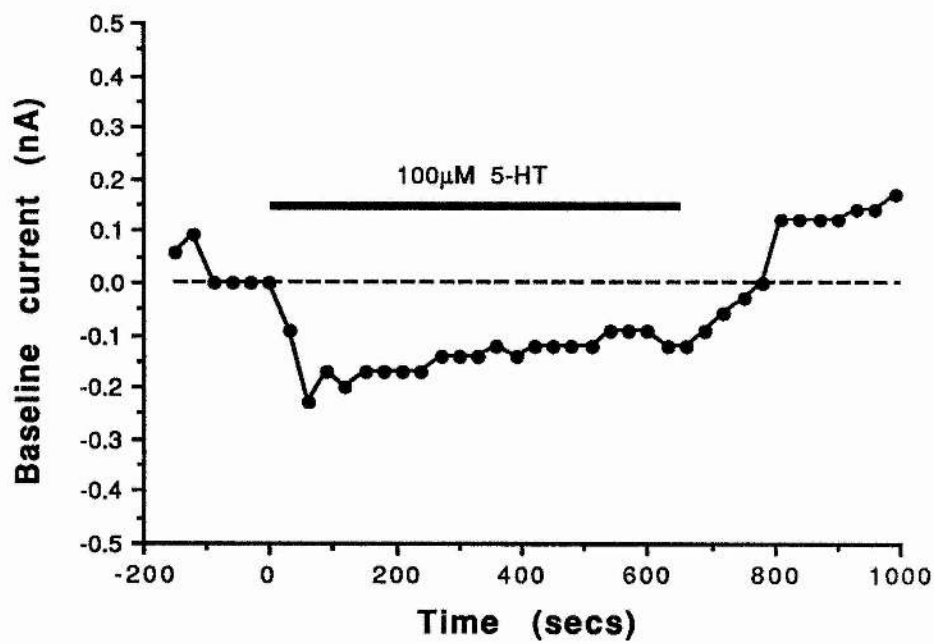
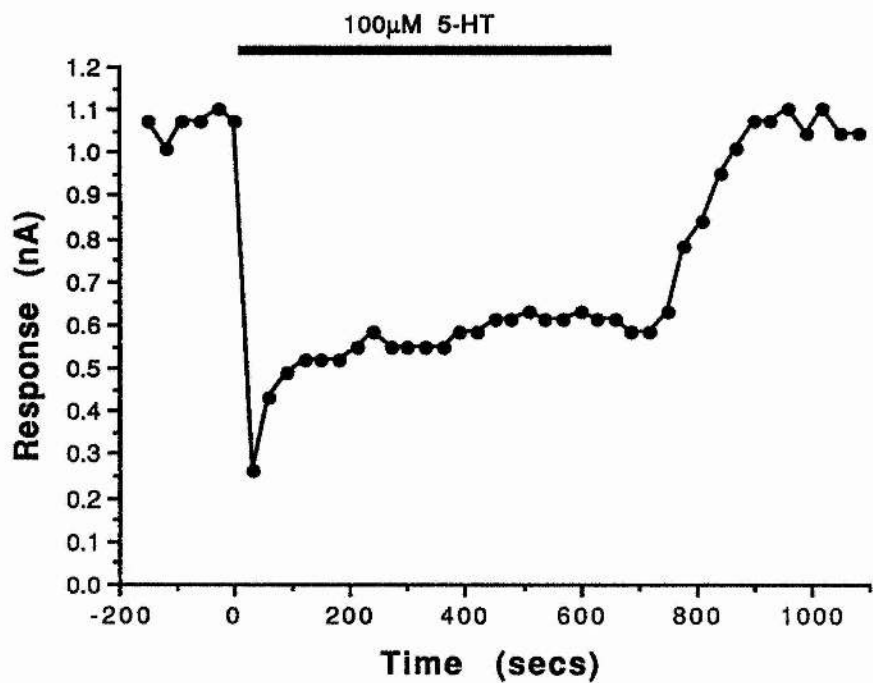
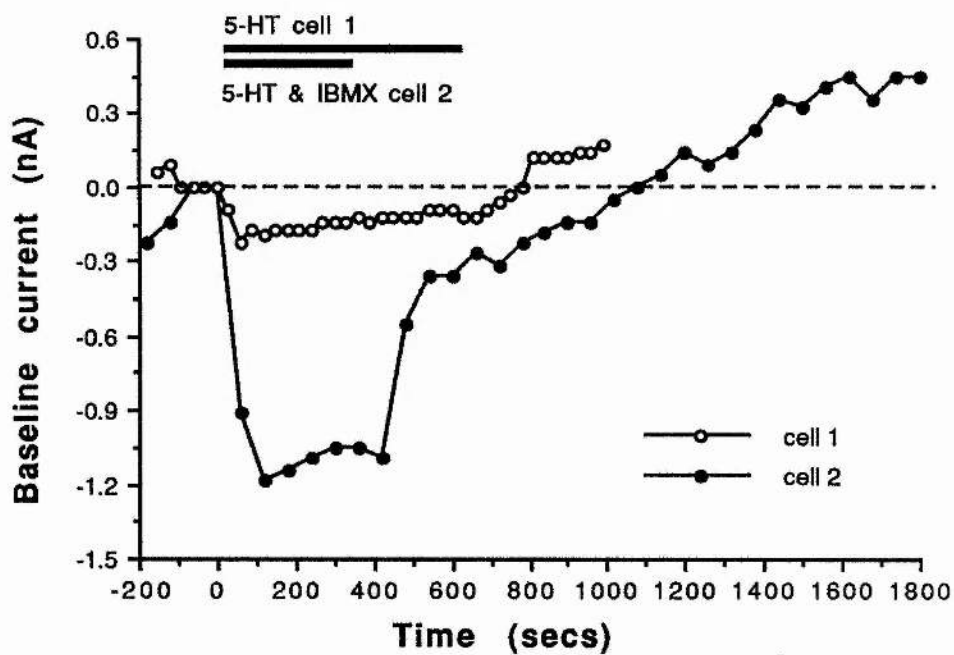
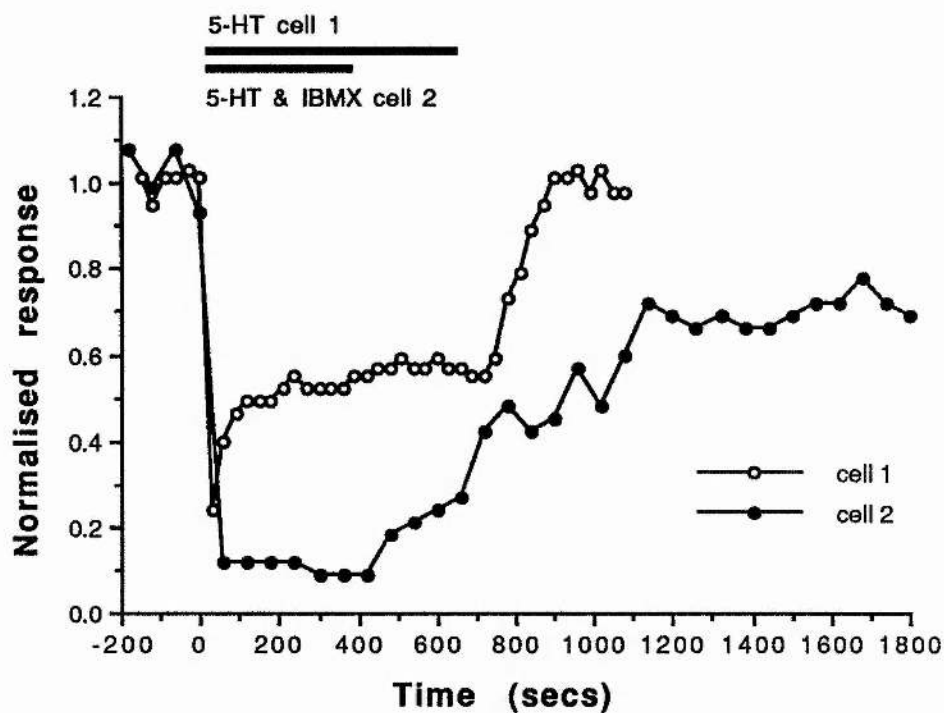


Figure 3.15

A comparison of the effects of $100\mu\text{M}$ 5-HT alone and $100\mu\text{M}$ 5-HT plus 1mM IBMX on the amplitude of the FMRFamide response and baseline current levels for C1 neurones voltage clamped at -45mV . In the presence of IBMX, 5-HT reduced the amplitude of the FMRFamide response to a greater extent and this effect was prolonged in comparison to 5-HT only (upper graph). The FMRFamide responses were normalised to make the comparison. The presence of IBMX also potentiated and prolonged the inward current (lower graph). Applications of 5-HT and 5-HT with IBMX are indicated by the bars in each graph. Cell 1 refers to the experiment where 5-HT alone was used while cell 2 was the experiment where 5-HT was applied with IBMX. The baseline current level at time zero is indicated by the dashed line in the lower graph.



transduction from various extracellular signals into neurones. The immediate products in this catalytic action, inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) act as second messenger molecules at the beginning of a two way transduction pathway involving the mobilization of Ca^{2+} from intracellular storage pools and activation of the protein kinase C enzyme family.

3.6.1 INOSITOL 1,4,5-TRISPHOSPHATE (IP_3)

Figure 3.16 graphically illustrates the result of an experiment where IP_3 , at concentration of 0.78mM, was pressure injected into a C1 neurone. The estimated intracellular concentration of IP_3 was 0.78 - 7.8 μ M (see Methods). No significant change in the baseline current level was observed up to 600 seconds (10 minutes) after injection (n=3). Since there was no mimic of the FMRFamide response, it suggested that IP_3 was not the second messenger underlying the FMRFamide response.

3.6.2 PROTEIN KINASE C

Physiological activation of protein kinase C by DAG can be mimicked by certain tumour promoting phorbol esters. These substances are active when applied extracellularly, a property which has made them extremely useful tools when studying the involvement of

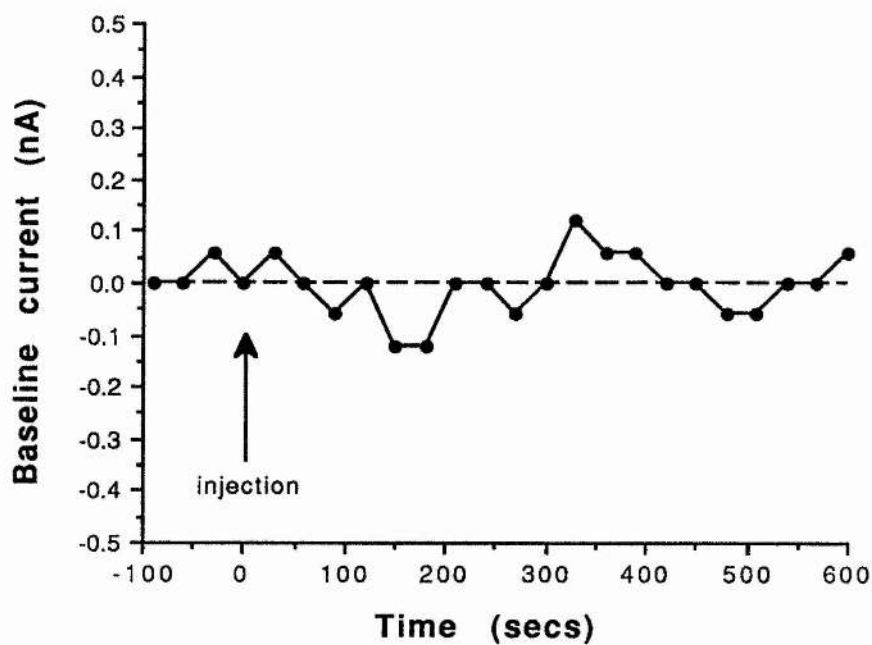


Figure 3.16

The lack of effect on baseline current level following the injection of 0.78mM inositol 1,4,5-trisphosphate (IP_3) into the cell body of a voltage clamped C1 neurone ($V_h -45mV$). The injection point (time zero) is marked by the vertical arrow and the original baseline current level at time zero is indicated by the dashed line.

protein kinase C in the opening and closure of ion channels.

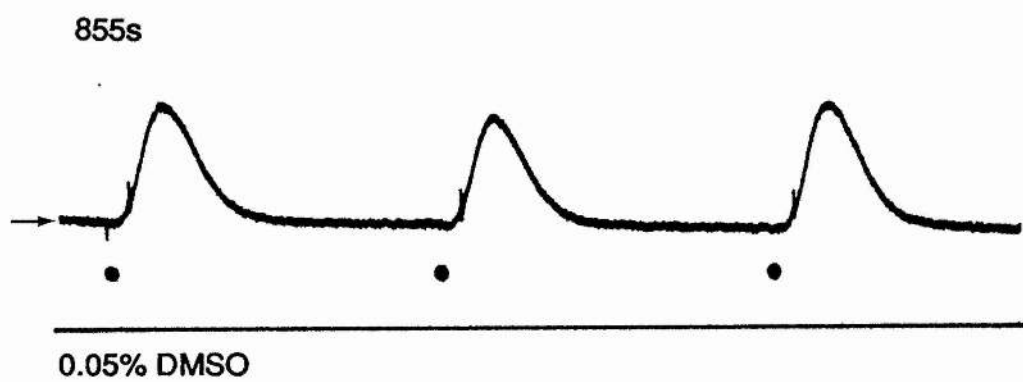
Due to their hydrophobicity, phorbol esters must first be dissolved in an organic solvent such as dimethylsulfoxide (DMSO). In these experiments, where phorbol 12,13-dibutyrate was applied to the C1 neurone, the final concentration of DMSO in the perfusing solution was 0.05% (v/v). The experimental record shown in Figure 3.17 indicates that when controls with 0.05% (v/v) DMSO were performed (n=3), there were no changes in the amplitude of the FMRFamide response or the baseline current level over a period of 945 seconds (15.75 minutes).

The result obtained with the application of 50 μ M phorbol 12,13-dibutyrate (n=3) is illustrated in Figure 3.18. Within 45 seconds of application, the amplitude of the FMRFamide response was reduced and an inward current developed. After 320 seconds (5.33 minutes) both effects appeared to have maximized. The preparation was then washed with normal saline. After washing for 240 seconds (4 minutes) the inward current had almost reduced to the original baseline current level, although the reduction in the FMRFamide response persisted. A longer washing period may have resulted in some recovery of the response. However, the neurones did not survive long enough to make this possible.

These results suggest that protein kinase C activation is not the mechanism which underlies the generation of the FMRFamide response in the C1 neurone,

Figure 3.17

The administration of 0.05% (v/v) DMSO dissolved in normal saline had no effect on the amplitude of the FMRFamide response or baseline current level of a C1 neurone, voltage clamped at -45mV. DMSO application is marked by the lines underneath the records. The time shown above each record refers to the first FMRFamide response in that record relative to the start of DMSO application (time zero). The arrows to the left of the records show the original position of the baseline current level at time zero. FMRFamide was applied at the points marked by the black dots.



V_h -45mV

0.5nA
10s

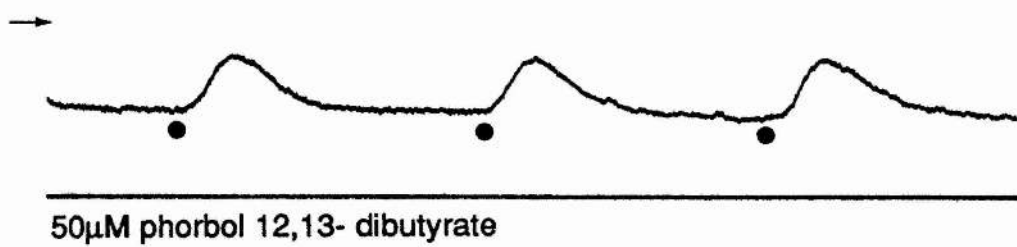
Figure 3.18

The influence of phorbol 12,13-dibutyrate on the amplitude of the FMRFamide response and baseline current level on a C1 neurone voltage clamped at -45mV. This substance at a concentration of 50 μ M dissolved in 0.05% (v/v) DMSO in normal saline produced an irreversible reduction of the amplitude of the FMRFamide response and an almost fully reversible inward current. The lines underneath the records mark where phorbol 12,13-dibutyrate was applied. The time marked above each record refers to the first FMRFamide response in that record relative to the start of application of phorbol 12,13-dibutyrate (time zero). The arrows to the left of the records show the original level of baseline current at time zero. FMRFamide was applied at the points marked by the black dots. The rapid inward deflections in the upper record were unclamped axon spikes.

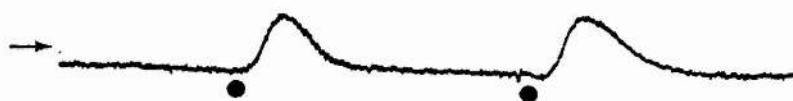
-80s



320s



680s



V_h -45mV

0.5nA
10s

but protein kinase C could be important as a modulator of the FMRFamide response.

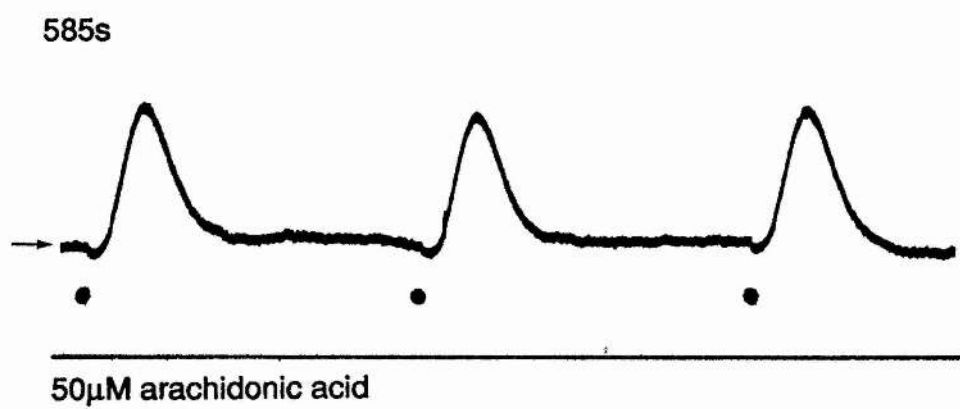
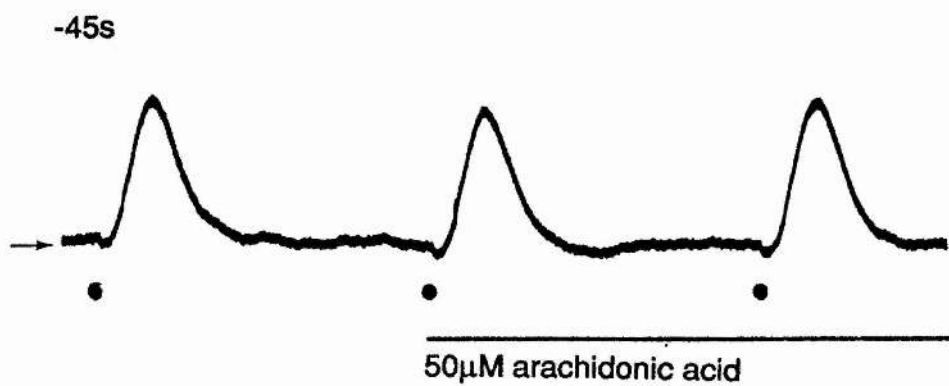
3.7 ARACHIDONIC ACID

Arachidonic acid is a polyunsaturated fatty acid which can be released from the esterified stores of membrane phospholipids by the action of Ca^{2+} requiring phospholipases. Arachidonic acid can then be metabolized to a number of biologically active oxidation products which are known as eicosanoids. One of the main breakdown pathways for arachidonic acid involves the lipoxygenase enzymes which produce leukotrienes, hydroxyeicosatetraenoic acids (HETE), hydroperoxyeicosatetraenoic acids (HPETE) and lipoxins. Piomelli et al, (1987) have provided evidence that the receptor-stimulated release of arachidonic acid and its metabolism through to 12-HPETE or subsequent products, mediates the inhibitory synaptic response of FMRFamide (opening "S" K^+ channels) in Aplysia sensory neurones. It was therefore important to investigate if a cascade involving arachidonic acid plays a role in the FMRFamide induced slow K^+ response in the C1 neurone of Helix aspersa.

50 μM arachidonic acid dissolved in 0.05% (v/v) DMSO was applied to a C1 neurone (Figure 3.19). Throughout the duration of the experiment, 675 seconds (11.25 minutes), the amplitude of the FMRFamide responses and the level of the baseline current remained stable (n=3). The data from the experiment are

Figure 3.19

Arachidonic acid at a concentration of $50\mu\text{M}$ dissolved in 0.05% (v/v) DMSO in normal saline did not influence the amplitude of the FMRamide response of a C1 neurone voltage clamped at -45mV . Baseline current level was also unaffected. Arachidonic acid application is marked by the lines underneath the records. The time indicated above each record refers to the first FMRamide response in that record relative to the starting time of arachidonic acid application (time zero). The arrows to the left of the records shows the position of the baseline current level at time zero. FMRamide was administered at the points marked by the black dots.



V_h -45mV

0.5nA
10s

plotted in Figure 3.20. Since there was no mimic of, and no effect on, the FMRFamide response, the release of arachidonic acid and the subsequent production of its metabolites do not appear to be involved in the response.

3.8 IS THE FMRFamide RESPONSE DEPENDENT ON Ca^{2+} ?

The opening of certain K^+ channels requires the presence of Ca^{2+} on the intracellular surface of the neuronal membrane. Ca^{2+} ions may enter the neurone through channels from the extracellular environment or originate from intracellular stores of Ca^{2+} . Since the discovery of Ca^{2+} dependent K^+ channels in molluscan neurones by Meech, (1978) they have been shown to be widely distributed in excitable cells.

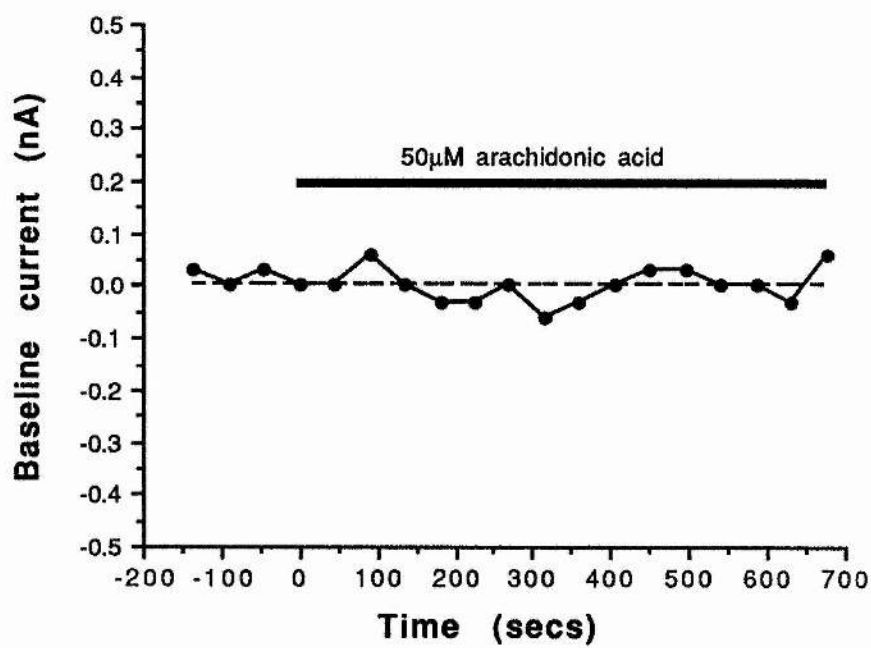
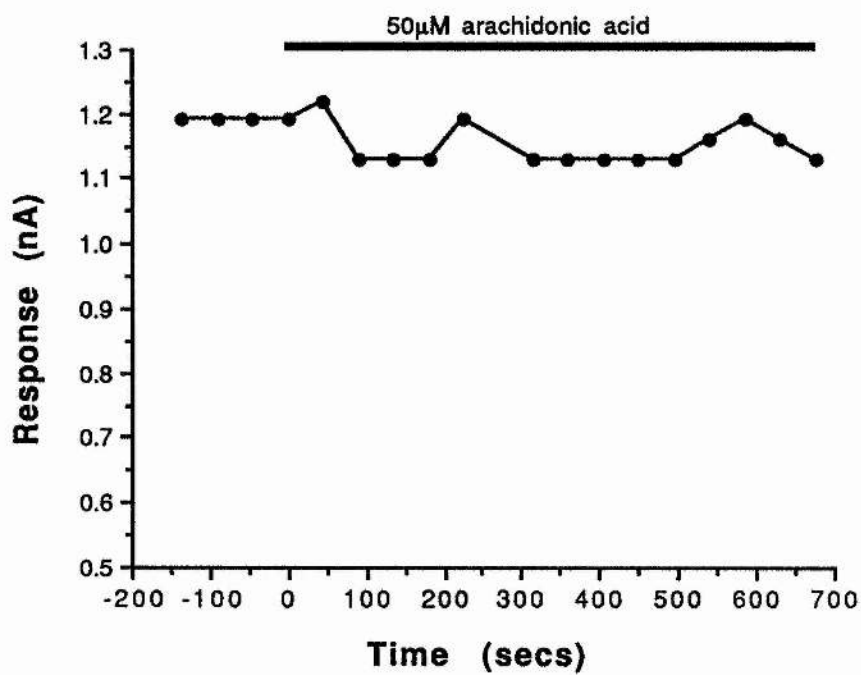
It was important to determine if the outward K^+ current induced by FMRFamide in the C1 neurone was Ca^{2+} dependent. Extracellular and intracellular Ca^{2+} concentrations were reduced by two different means described below. One or both of these approaches have been commonly utilized by other groups (Hammond et al 1987, Sawada et al 1987, Paupardin-Tritsch and Gerschenfeld 1990).

3.8.1 THE EXTRACELLULAR DEPLETION OF Ca^{2+}

The extracellular free Ca^{2+} concentration was effectively reduced to zero by perfusing the preparation with a modified saline where Mg^{2+} were

Figure 3.20

Data showing the lack of effect of $50\mu\text{M}$ arachidonic acid on the amplitude of the FMRFamide response (upper graph) or baseline current level (lower graph) for a C1 neurone voltage clamped at -45mV . The bars mark where $50\mu\text{M}$ arachidonic acid was applied. The level of the baseline current at time zero is marked by the dashed line on the lower graph.



substituted for Ca^{2+} and the Ca^{2+} chelator EGTA was added (80mM NaCl, 5mM KCl, 9.5mM MgCl_2 , 2mM EGTA, 20mM Hepes, pH 7.5).

The recordings in Figure 3.21 show that when the Ca^{2+} saline was applied to a C1 neurone, there was no change in the amplitude of the FMRFamide response over an 810 second (13.5 minute) period ($n=4$). Thus it appeared that the entry of Ca^{2+} into the neurone was not required for the production of the FMRFamide response.

The removal of external Ca^{2+} did induce an inward current, which was almost fully reversed on washing the preparation with normal saline (Figures 3.21 and 3.22, $n=4$). This inward current effect was not investigated further. However, it did suggest that Ca^{2+} dependent K^+ channels may normally be open at the holding potential of -45mV, although none were observed in patch clamp experiments.

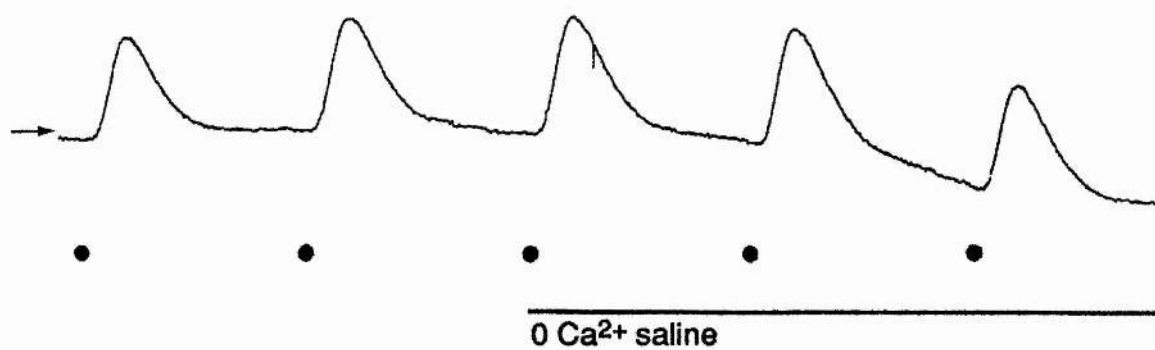
3.8.2 REDUCTION IN INTRACELLULAR FREE Ca^{2+} CONCENTRATION

To further evaluate any possible contribution of Ca^{2+} in the FMRFamide response, intracellular Ca^{2+} concentration was reduced by injecting the Ca^{2+} chelator EGTA into the cell body of the C1 neurone. EGTA was injected at a concentration of 700mM, which would have given an intracellular concentration of 0.7-7mM (see Methods). The EGTA itself was dissolved in a buffer solution which consisted of 100mM KCl, 20mM

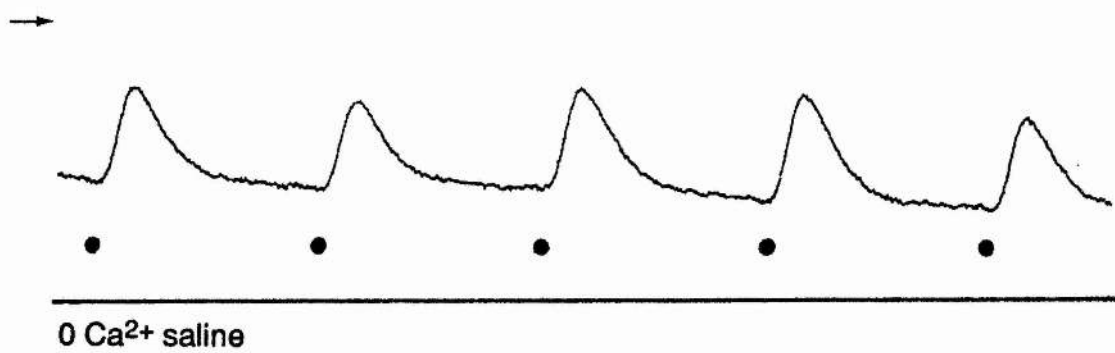
Figure 3.21

An experiment to show that OCa^{2+} saline had no effect on the amplitude of the FMRFamide response but did induce an almost fully reversible inward current in a C1 neurone voltage clamped at -45mV . Application of OCa^{2+} saline is marked by the lines underneath the records. The time marked above each record refers to the first FMRFamide response in that record relative to the start of OCa^{2+} application (time zero). The arrows to the left of the records show the position of the baseline current level at time zero. FMRFamide was applied at the points marked the black dots.

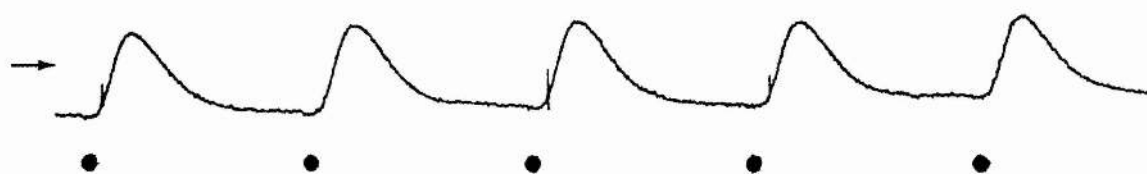
-60s



570s



1050s

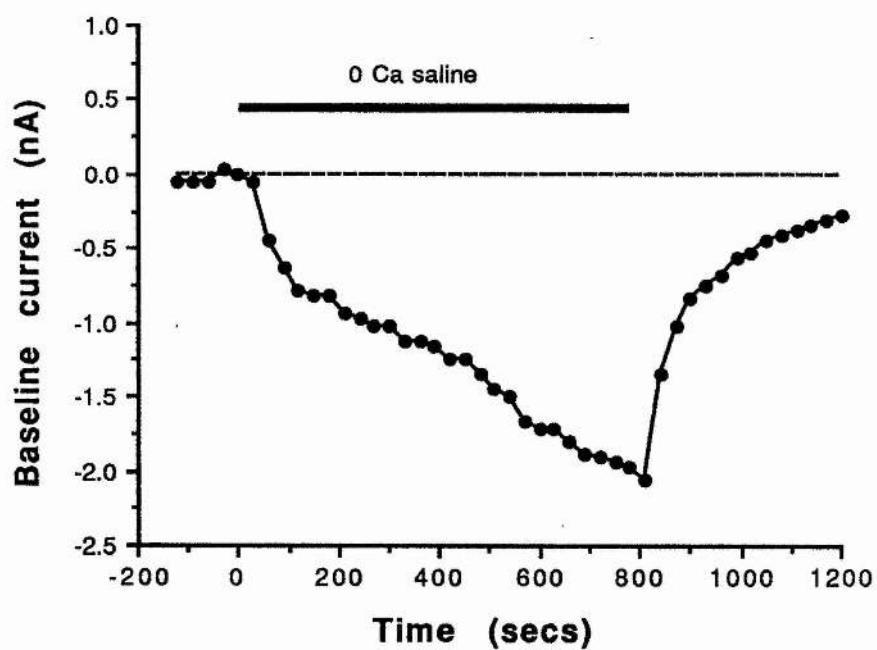
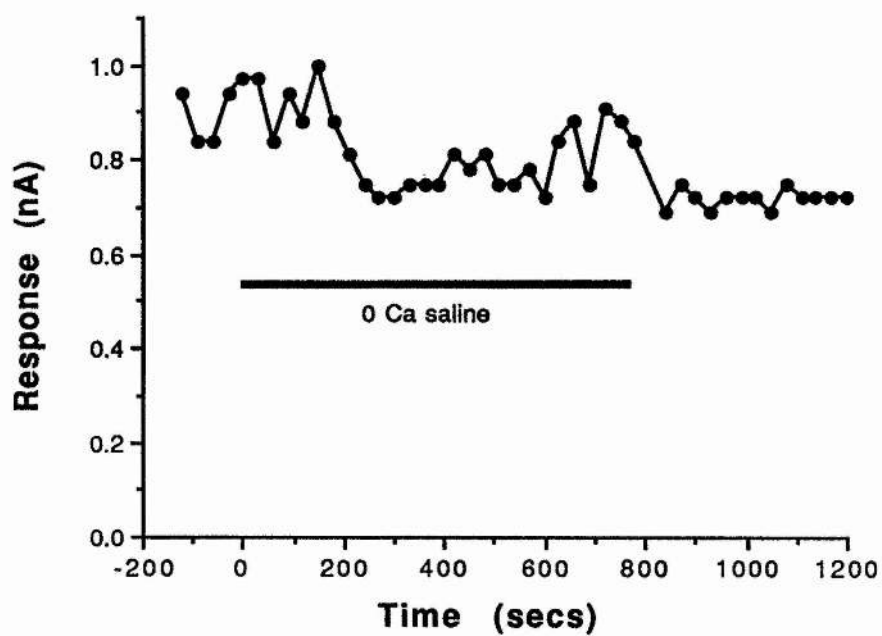


V_h -45mV

0.5nA
10s

Figure 3.22

Data showing the lack of effect of OCa^{2+} saline on the amplitude of the FMRFamide response of a C1 neurone voltage clamped at -45mV (upper graph). It did however, induce a reversible inward current (lower graph). Application of OCa^{2+} saline is marked by the bar on each graph. The baseline current level at time zero is indicated by the dashed line on the lower graph.



Hepes, 1% (w/v) fast green, pH 7.4 with KOH. This buffer solution was the same as that used with GTP γ S injection. It had no effect on the FMRFamide response and induced a small inward current (Figure 3.3).

The data from an experiment where EGTA was injected into a C1 neurone are plotted in Figure 3.23. It shows that after a 1800 second (30 minute) time period, there was no reduction in amplitude of the FMRFamide response (n=3). This is another indication that Ca^{2+} are not required for the production of the FMRFamide response.

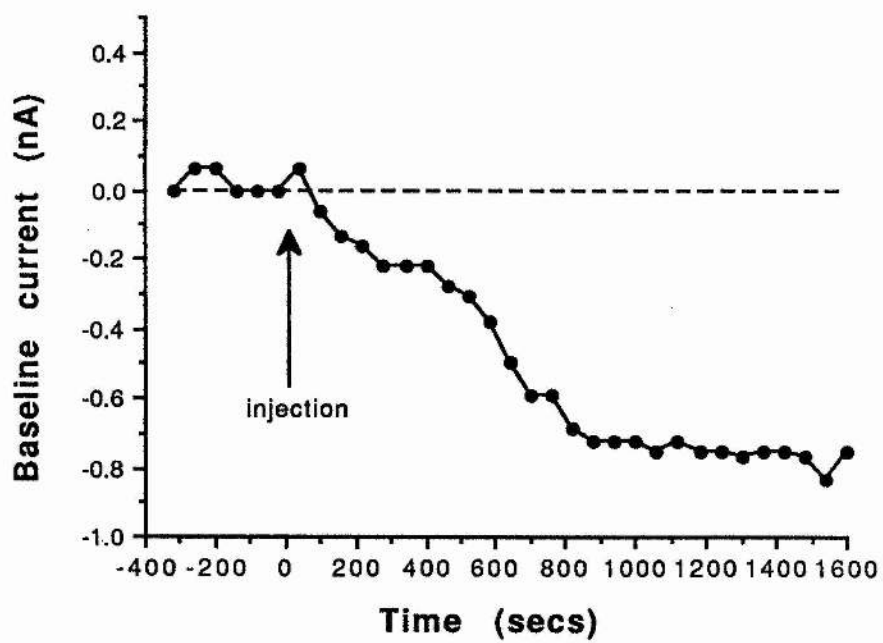
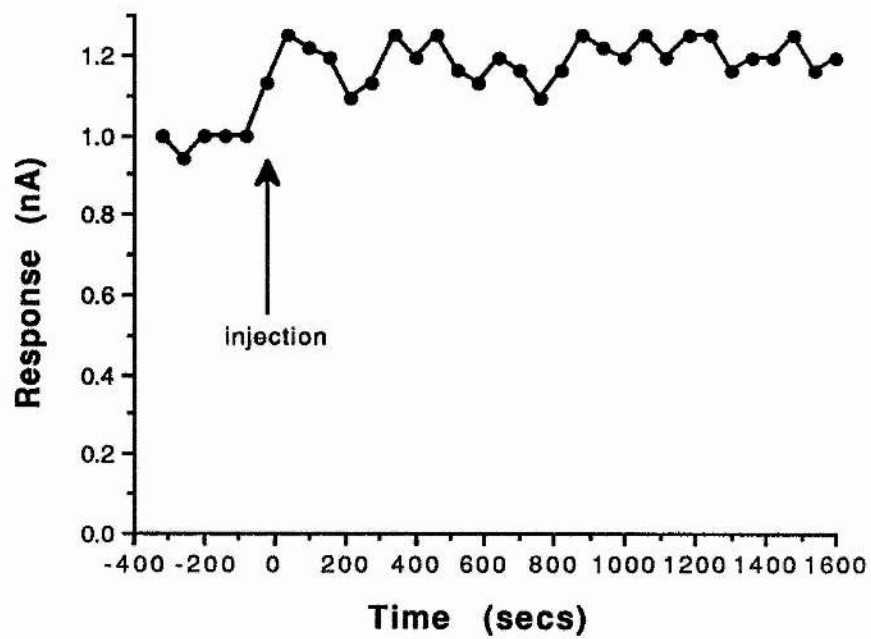
Injection of EGTA did however, induce an inward current (approximately 0.8nA, n=3) which was much larger than that caused by the buffer solution alone (approximately 0.1nA), again implying some inhibition of background activity of Ca^{2+} activated K^{+} channels (as previously suggested (3.8.2)). It was not investigated further.

3.9 PROTEIN PHOSPHATASE INHIBITION BY OKADAIC ACID

Protein phosphatase enzymes have the role of dephosphorylating cellular proteins. The experiments described so far have been focussed on the activation of protein kinases by second messengers and a phorbol ester to increase protein phosphorylation levels. An alternative approach is to inhibit protein phosphatases. This has been made possible with the availability of substances such as okadaic acid.

Figure 3.23

EGTA injection at a concentration of 700mM into the cell body of a C1 neurone voltage clamped at -45mV had no significant effect on the amplitude of the FMRFamide response (upper graph). It did induce an irreversible inward current (lower graph). EGTA injection is indicated by the vertical arrow on each graph. The baseline current level at time zero is indicated by the dashed line in the lower graph.



Okadaic acid is a polyether fatty acid which was first isolated from the marine sponges Halichondria okadaei and Halichondria melanodocia (Tachibana et al, 1981). It was shown to be produced by several types of dinoflagellates and to be concentrated in filter feeding marine sponges. Clear evidence has indicated that okadaic acid is a very potent inhibitor of protein phosphatases in the cytosol of mammalian cells that dephosphorylate serine and threonine residues (Takai et al, 1987). Protein phosphatases 1 and 2A are both sensitive to okadaic acid. Of the other two major protein-serine/ threonine phosphatases, the Ca^{2+} /calmodulin-dependent protein phosphatase 2B is markedly less sensitive, while the Mg^{2+} dependent protein phosphatase 2C is unaffected (Biolojan and Takai, 1988). Since protein phosphatases 1, 2A and 2B have been identified in invertebrates (Orgad et al, 1987; Pondaven and Cohen, 1987), it seemed reasonable to assume that okadaic acid would inhibit protein phosphatases 1 and 2A in the C1 neurone. Okadaic acid was originally dissolved in 100% DMSO and then diluted in normal saline to a concentration of $1\mu\text{M}$ (0.01% DMSO (v/v)). Figure 3.24 shows the result of an experiment where $1\mu\text{M}$ okadaic acid was applied to a C1 neurone. After a 1200 second (20 minute) exposure to okadaic acid the amplitude of the FMRFamide response was reduced to approximately 15% of its original amplitude ($n=3$). This suggests that the FMRFamide response involves protein phosphorylation and that protein

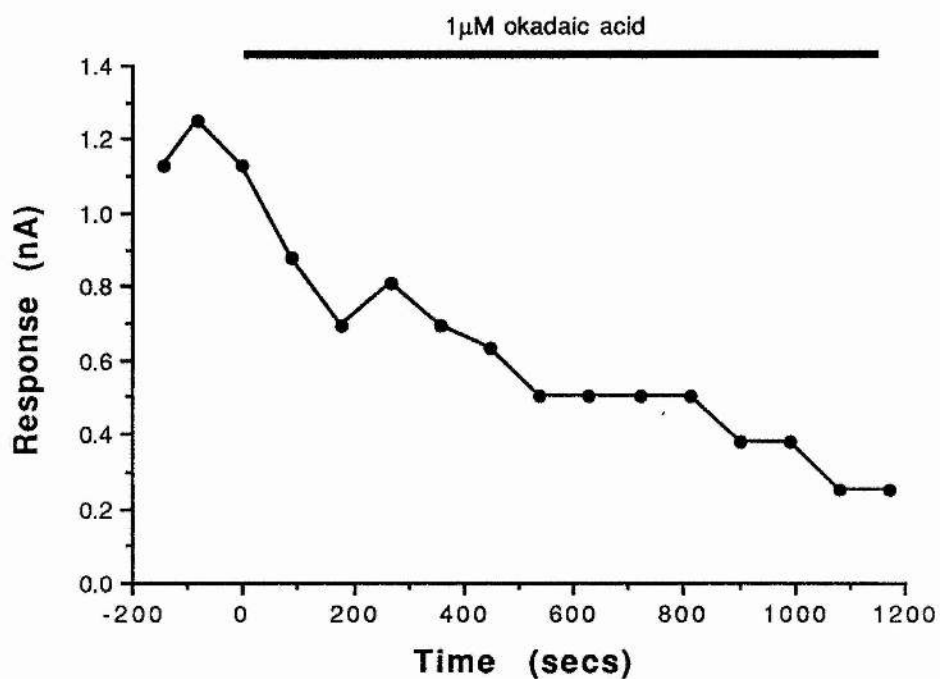


Figure 3.24

Okadaic acid at a concentration of $1\mu\text{M}$ dissolved in 0.01% (v/v) DMSO in normal saline reduced the amplitude of the FMRFamide response of a C1 neurone voltage clamped at -45mV . Okadaic acid application is marked by the bar.

phosphatases 1 and/or 2A may play a role in the dephosphorylation process.

3.10 SINGLE CHANNEL RECORDINGS

Recordings were made from cell attached patches on the C1 neurone. This procedure was useful since FMRFamide application out with the patch electrode could only have access to any ion channels within the patch of membrane via the production of an intracellular second messenger.

The term patch potential (V_p) is used to refer to the potential across the patch of membrane. The patch potential was calculated by subtracting the potential inside the pipette (V_{pipette}) from the resting membrane potential (V_m) of the neurone:

$$V_p = V_m - V_{\text{pipette}}$$

V_m was taken as -50mV since this was the average value observed from the intracellular recording experiments. Also, in cell attached patch experiments, where the membrane was inadvertently ruptured in an attempt to obtain a seal, the measured resting membrane potential was usually equal or close to -50mV.

With normal saline in the patch pipette (5mM K^+) unitary outward currents were observed at depolarized patch potentials. Only on one occasion were unitary outward currents seen at patch potentials close to the

resting membrane potential (1 out of 10 patches). The records from this patch are shown in Figure 3.25. The unitary current amplitudes are plotted against patch potential for this patch in Figure 3.26. The curve shown on the graph is a fit of the data points to the theoretical relationship from the GHK equation (Hodgkin and Katz, 1949) for a K^+ current, using the mean permeability value calculated from the data points. The mean permeability value was $1.68 \times 10^{-13} \text{cm}^3 \text{s}^{-1}$. The intracellular concentration of K^+ was taken as 98mM (Alvarez-Leefmans and Gamino, 1982). The reversal potential of the relationship was approximately -70mV which is close to that predicted by the Nernst equation (-74mV) assuming that the intracellular K^+ concentration is 98mM.

When the K^+ concentration in the patch pipette was reduced to 1mM, the unitary outward currents became larger (at V_p -20mV, 0.92pA with 1mM K^+ compared to 0.78 pA with 5mM K^+) as would have been expected for a current carried by K^+ . Records of unitary currents made in such circumstances are shown in Figure 3.27 and again the current/voltage relationship is plotted with the theoretical curve for the GHK equation in Figure 3.28. The average permeability constant calculated from the data points was $1.62 \times 10^{-13} \text{cm}^3 \text{s}^{-1}$. This value is close to $1.68 \times 10^{-13} \text{cm}^3 \text{s}^{-1}$ with 5mM K^+ in the patch pipette which indicates the same channel type being opened under both recording conditions.

When FMRFamide was applied to the neurones, the patch pipettes were filled with 1mM K^+ saline so that

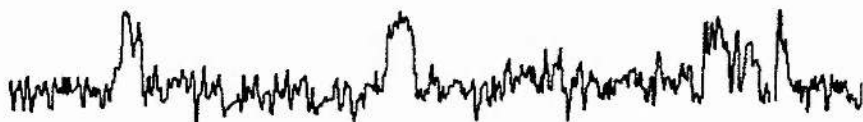
Figure 3.25

Records of unitary currents obtained from a cell attached patch on a C1 neurone held at various patch potentials. The patch potentials used are marked above the appropriate section of record. The recording solution in the patch pipette was normal saline which contained 5mM K^+ .

$V_p - 50\text{mV}$



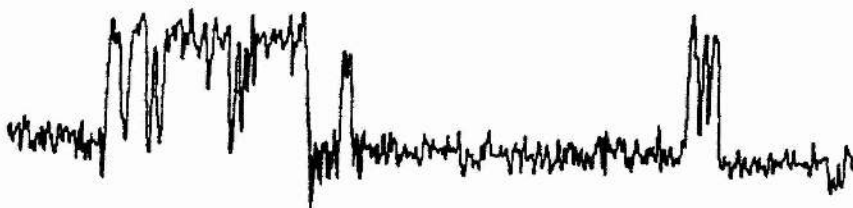
$V_p - 40\text{mV}$



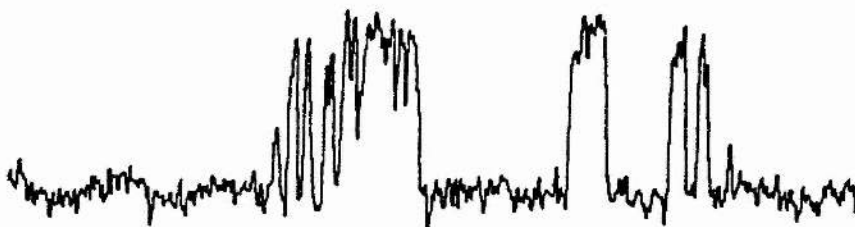
$V_p - 30\text{mV}$



$V_p - 20\text{mV}$



$V_p - 10\text{mV}$



1pA

40ms

Figure 3.26

Current /voltage relationship of the unitary currents obtained from a cell attached patch on the C1 neurone with 5mM K^+ in the patch pipette. The line drawn through the points represents the theoretical relationship based on the GHK equation for a K^+ current. The permeability value of $1.68 \times 10^{-13} \text{cm}^3 \text{s}^{-1}$ was calculated from the data points, assuming an intracellular K^+ concentration of 98mM.

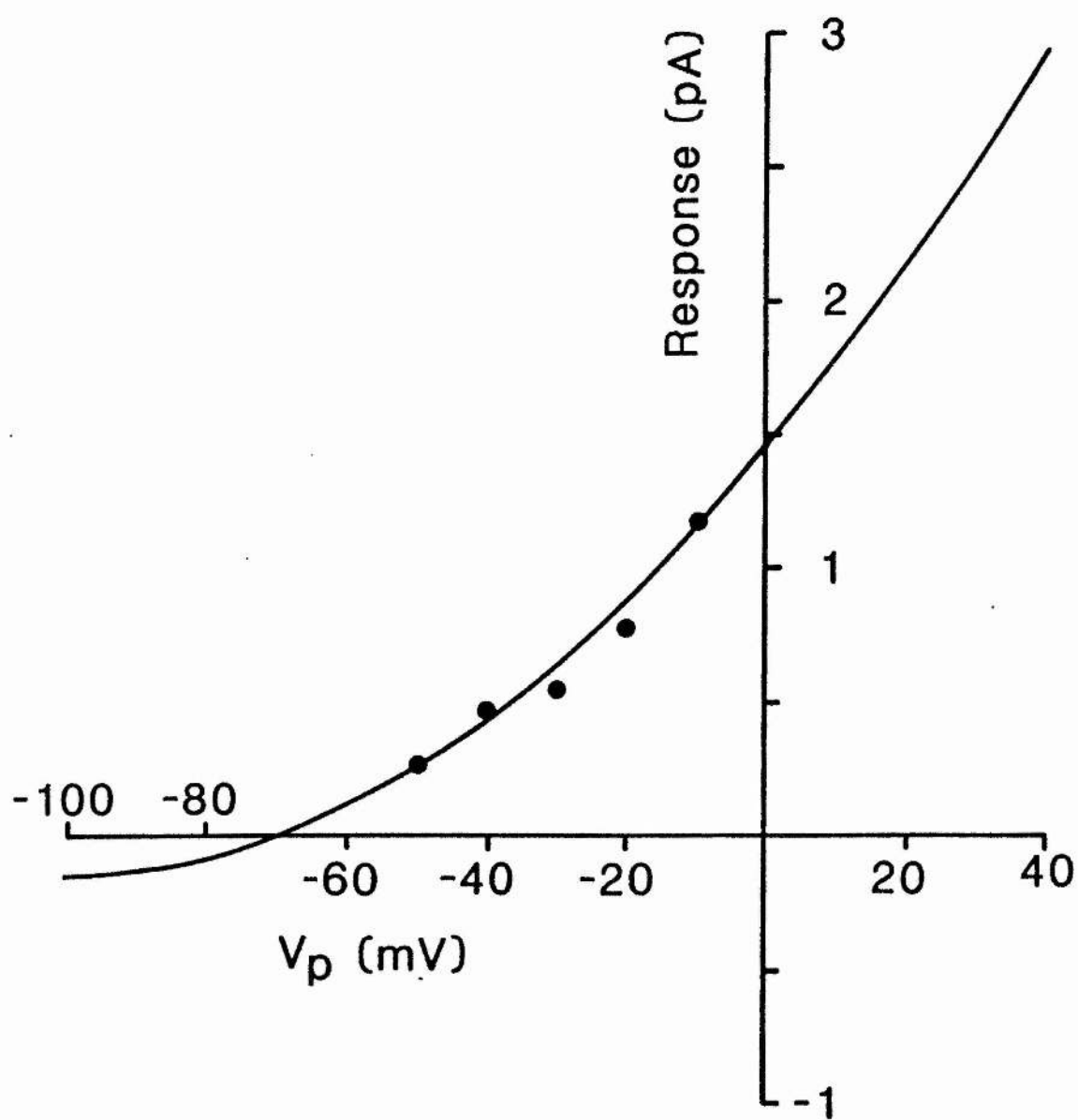


Figure 3.27

Records of unitary currents obtained from a cell attached patch on a C1 neurone held at various patch potentials. The patch potentials used are marked above the appropriate section of recording. The patch pipette recording solution was a modified saline which contained 1mM K^+ .

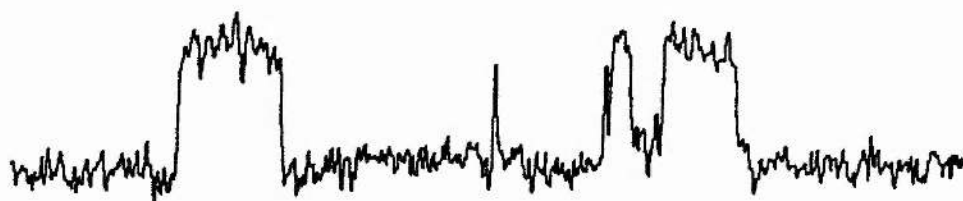
$V_p - 50\text{mV}$



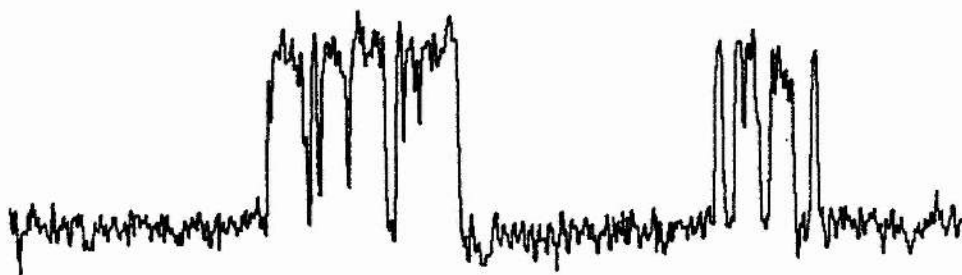
$V_p - 30\text{mV}$



$V_p - 20\text{mV}$



$V_p - 10\text{mV}$



$V_p + 10\text{mV}$

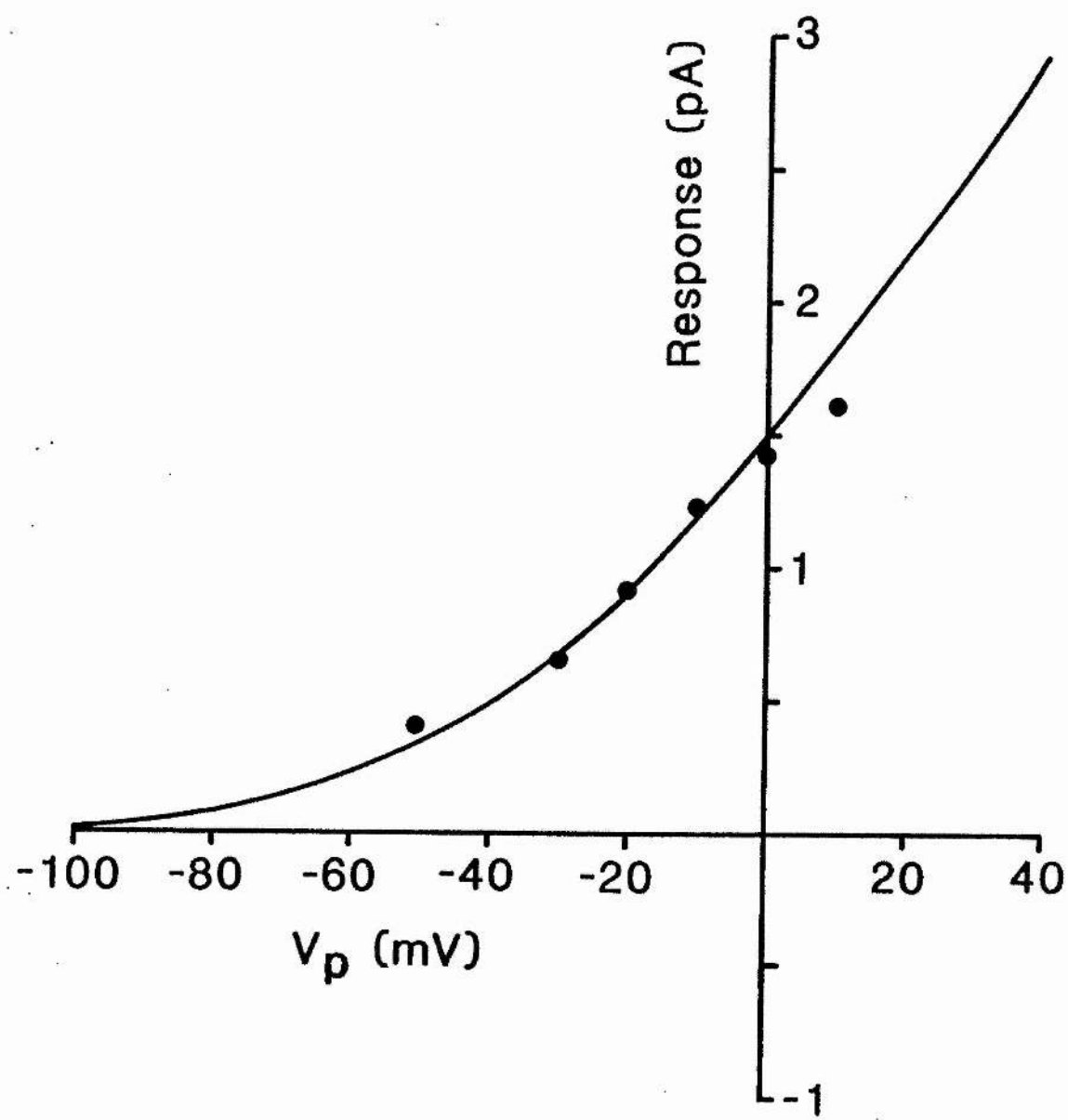


1pA

40ms

Figure 3.28

Current /voltage relationship of the unitary currents obtained from a cell attached patch on the C1 neurone with 1mM K^+ in the patch pipette. The line drawn through the points represents the theoretical relationship based on the GHK equation for a K^+ current. The permeability value of $1.62 \times 10^{-13} \text{cm}^3 \text{s}^{-1}$ was calculated from the data points assuming an intracellular K^+ concentration of 98mM.



any channels which may have been observed at a patch potential of -45mV (holding potential used in intracellular recording experiments) would be larger and easier to recognise. Figure 3.29a is a typical example of a recording where FMRFamide was applied to a C1 neurone outwith the membrane patch ($n=30$). Patches were obtained from several regions of the C1 neurone cell body. These patches showed no channel activity before or after the application of FMRFamide. FMRFamide was then washed from the preparation. A concern when FMRFamide did not induce channel activity was that no K^+ channels were actually present in that particular patch. To check if this was the case, the membrane patches were depolarized and with most of them (Figure 3.29b) the presence of K^+ channels became apparent. Therefore FMRFamide application at a patch potential of -45mV did not open K^+ channels or influence the activity of the K^+ channels which had a permeability value of $1.62 \times 10^{-13} \text{cm}^3 \text{s}^{-1}$.

3.11 THE FAST DEPOLARIZING FMRFamide RESPONSE OF THE C2 NEURONE

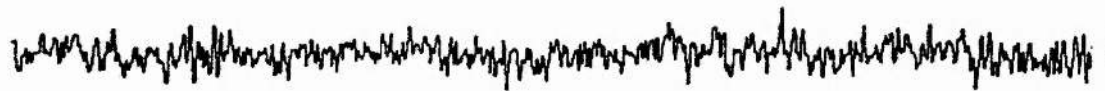
Fast depolarizing Na^+ current responses induced by FMRFamide have been found in three identified Helix aspersa neurones, namely the visceral E13, the right parietal F2 and the cerebral C2 neurones (Cottrell et al, 1984; Cottrell and Davies, 1987; Cottrell et al, 1990). The response in the C2 neurone to FMRFamide was quite unique for a neuropeptide because it appeared to

Figure 3.29 a) and b)

Records from a cell attached patch on a C1 neurone showing that FMRFamide at a concentration of $100\mu\text{M}$, did not open channels at a patch potential of -45mV . The recording solution in the patch pipette contained a modified saline with 1mM K^+ concentration. a) The patch potential was held at -45mV . The time marked above three of the four sections of record is relative to the start of the application of FMRFamide (time zero). The second from top record shows the point at which FMRFamide was applied. The voltage record underneath shows that FMRFamide was applied by a pressure pulse which lasted 40ms . b) Unitary current record from the same cell attached patch at a depolarized patch potential of -20mV .

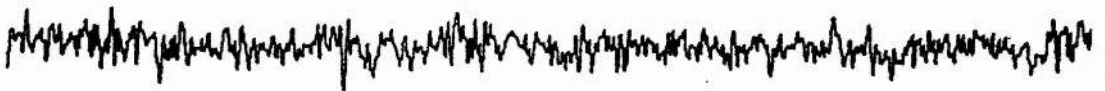
a) V_p -45mV

-227ms

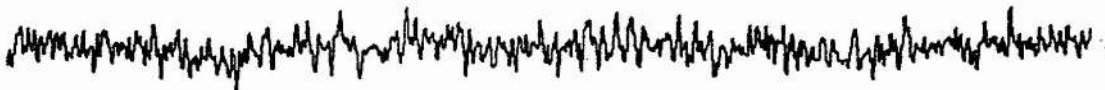


FMRFamide

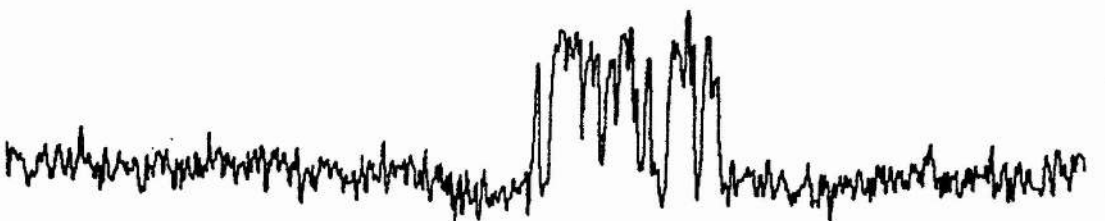
114ms



4.5s



b) V_p -20mV



1pA

40ms

be due to the direct activation of a ligand gated Na^+ channel (Cottrell *et al*, 1990). The purpose of the experiments described in the following sections was to further characterize this fast response.

3.11.1 GENERAL CHARACTERISTICS OF THE FAST Na^+ RESPONSE IN THE C2 NEURONE

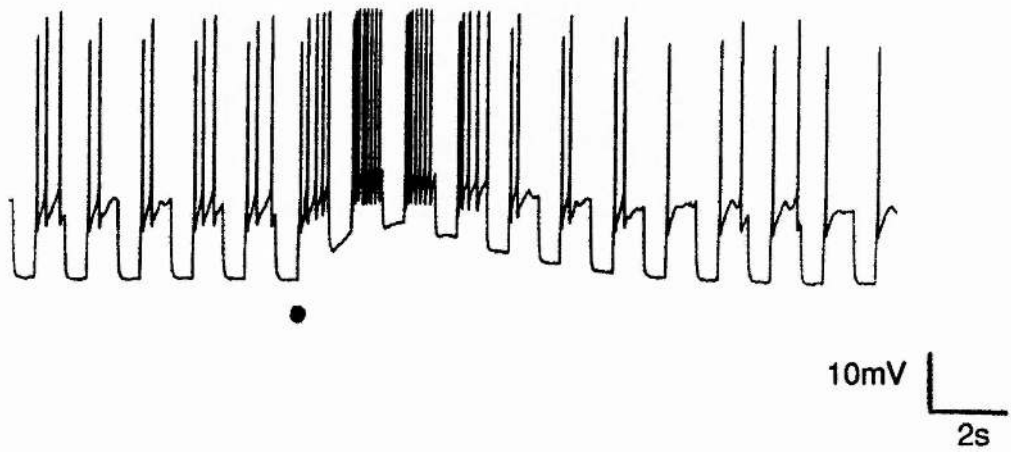
The depolarizing FMRFamide response of the C2 neurone is relatively fast. This can be appreciated when experimental records are examined. Figures 3.30a and b show the response under current clamp and voltage clamp conditions. The average resting membrane potential of 40 neurones was -47mV . The average time taken between FMRFamide application and the maximum amplitude of the inward current response was approximately 2.5 seconds (Figure 3.30b). Since this response was appreciably faster than the slow hyperpolarizing K^+ response of the C1 neurone (average 7 seconds) an expanded time scale was used.

When current clamped, the C2 neurone spiked continuously. This feature was used to identify the neurone if there was any doubt of its position in the ganglion. On most occasions the neurone was located adjacent to the C1 neurone (Figure 2.2). With the addition of FMRFamide, the neurone was depolarized, an increase in spike frequency was observed and input resistance was reduced as indicated by the reduction in the size of the voltage deflections (Figure 3.30a).

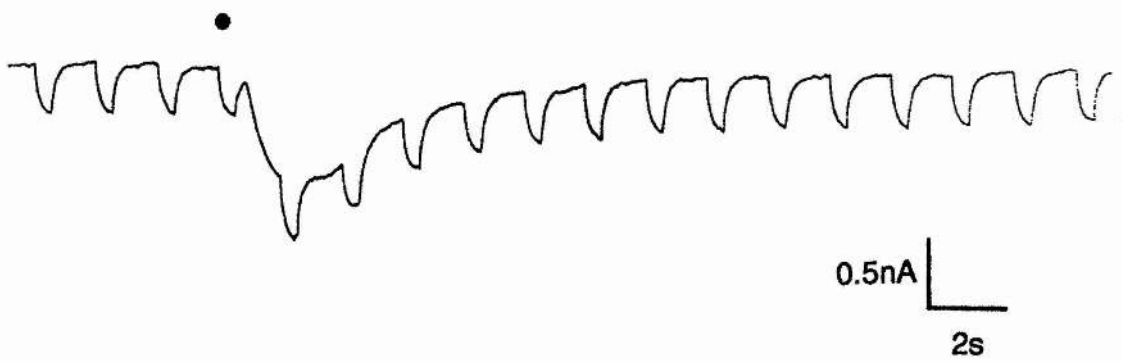
Figure 3.30 a), b) and c).

The fast depolarizing response induced by FMRFamide in the C2 neurone under current and voltage clamp conditions. a) Current clamp recording of a neurone with a resting membrane potential (V_m) of -50mV. The addition of FMRFamide caused a depolarization, an increase in the spike frequency and the amplitude of the voltage deflections initiated by constant hyperpolarizing current pulses was reduced. This was an indication of decreased input resistance. b) Voltage clamp recording from the same neurone at a holding potential of -75mV (V_h). FMRFamide application induced an inward current associated with a decreased input resistance as indicated by the increased size of the current deflections initiated by a constant hyperpolarizing voltage step. c) Voltage clamp recording of a neurone which was held at -75mV. FMRFamide applications which were made in rapid succession desensitized the response. In a), b) and c) FMRFamide application is marked by the black dots.

a) $V_m - 50\text{mV}$



b) $V_h - 75\text{mV}$



c) $V_h - 75\text{mV}$

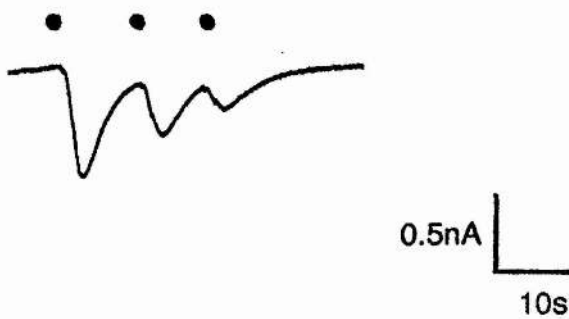


Figure 3.30c shows that repeated applications of FMRFamide over a short period of time resulted in the desensitization of the response.

The FMRFamide response/voltage relationship of the C2 neurone is shown in Figure 3.31. The amplitude of the inward current responses became larger at more negative potentials and smaller at more depolarized potentials. The relationship of current response against voltage was not linear but inwardly rectifying as would be expected from the constant field equation. This describes the movement of an individual ionic species across the membrane by simple diffusion along a constant potential gradient. Data points on the curve were only obtained up to a holding potential of -5mV. This was because any further depolarization resulted in the appearance of interfering axon spikes, voltage activated K^+ currents and high frequency oscillations of the voltage clamp. However, the values obtained suggested that the reversal potential of the response occurred at a more depolarized potential than 0mV (Figure 3.31).

3.12 CHARACTERIZATION OF THE FMRFamide INDUCED FAST DEPOLARIZING RESPONSE OF THE C2 NEURONE.

3.12.1 $0Na^+$ SALINE

C2 neurones were voltage clamped at -75mV (more hyperpolarized than resting membrane potential) to obtain larger responses and FMRFamide was applied every

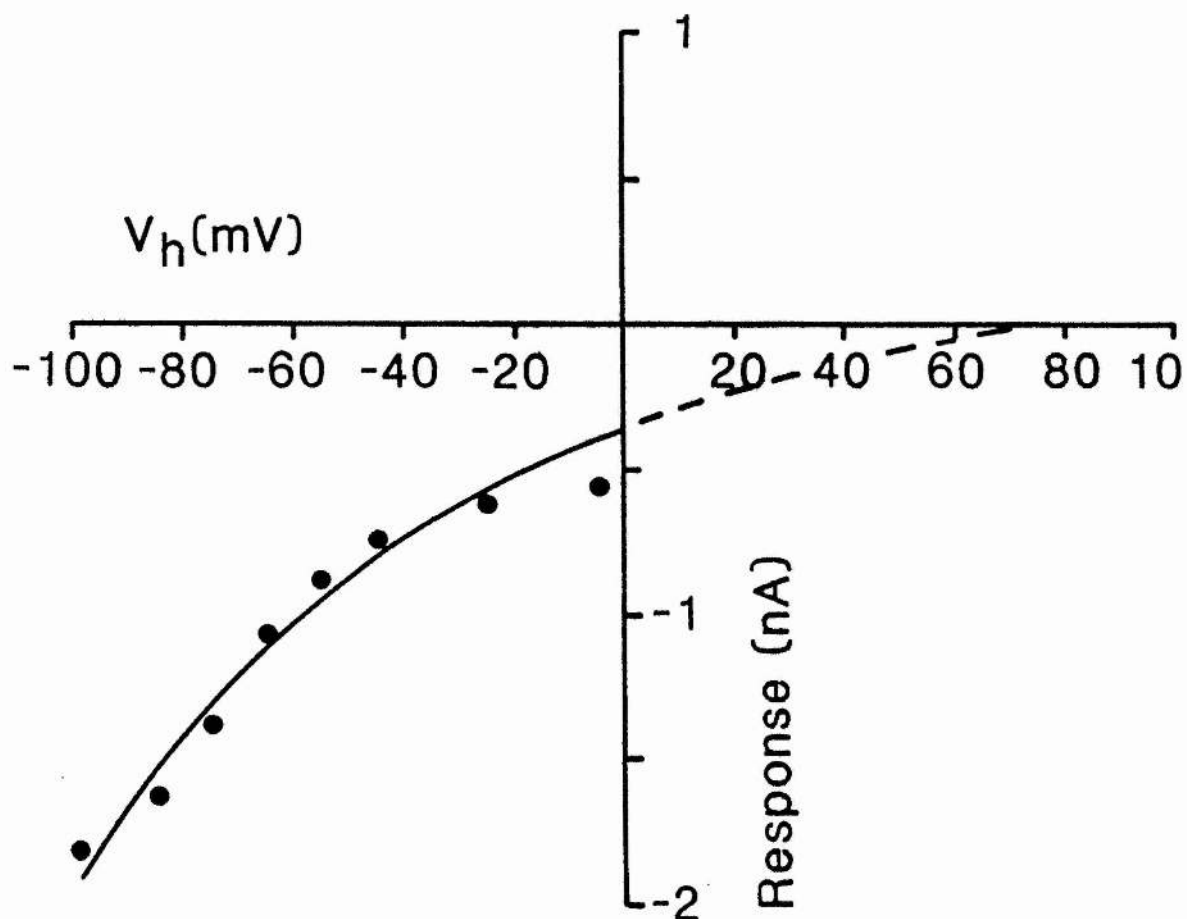


Figure 3.31

A plot of the current reponse/voltage relationship of the fast depolarizing FMRFamide response in the C2 neurone.

30 seconds. The upper experimental recording in Figure 3.32 shows that FMRFamide responses of regular amplitudes were obtained. The neurone was then exposed to a perfused saline to which no NaCl had been added (the NaCl had been replaced by sucrose, therefore it was termed as a ONa^+ saline). Within 60 seconds of exposure to the ONa^+ saline, the FMRFamide response had been reduced considerably (0.95nA to 0.19nA). When the neurone was exposed to normal saline again, the response recovered although not back to its original amplitude ($n=3$). The data of the experiment are plotted in Figure 3.33. This result confirmed that Na^+ was the main charge carrier for the FMRFamide response.

3.12.2 CoCl_2

CoCl_2 was applied to the C2 neurone as a simple method to block Ca^{2+} channels (Akaike *et al*, 1981) and hence determine if Ca^{2+} had a role in the production of the FMRFamide response. Figure 3.34 shows a recording where a C2 neurone held at -75mV was responding constantly to FMRFamide. Exposure of the neurone to 1mM CoCl_2 had no effect on the amplitude of the response ($n=3$). When the response amplitude is plotted against time (Figure 3.35) it can be clearly seen that 1mM CoCl_2 had no effect even after a 600 second (10 minutes) application. Therefore, a possible involvement of Ca^{2+} in the response was eliminated.

Figure 3.32

The application of ONa^+ saline reduced the amplitude of FMRFamide responses obtained from a C2 neurone held at -75mV . The response reversed to some extent on washing with normal saline. The time marked above each record refers to the first FMRFamide response in that record relative to the start of ONa^+ saline application (time zero). FMRFamide was applied at the points marked by the black dots.

-90s



60s



0 Na⁺ saline

240s



V_h -75mV

0.5nA
10s

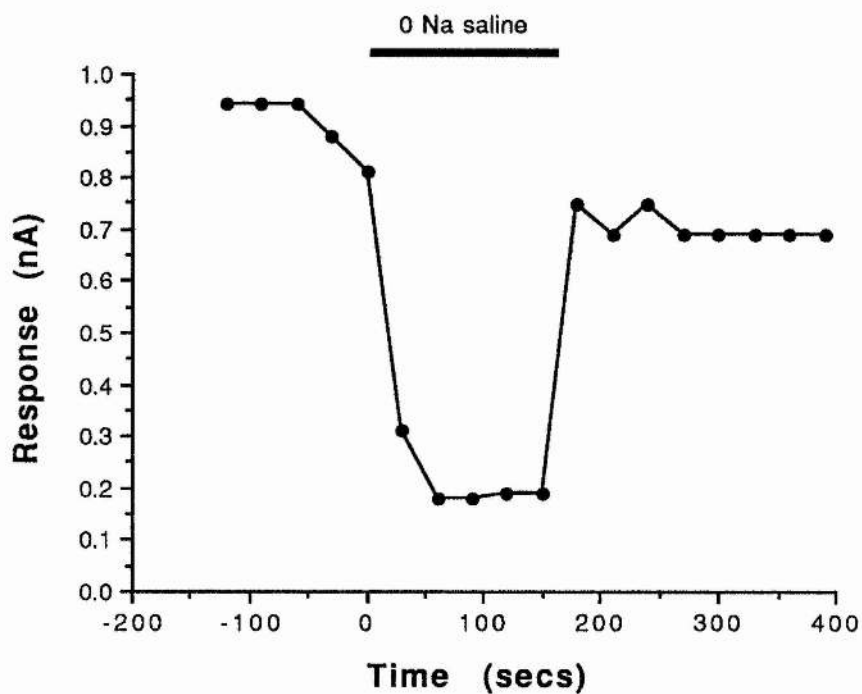


Figure 3.33

Data showing the effect of applying 0Na^+ saline to a C2 neurone voltage clamped at -75mV . This saline reduced the amplitude of the FMRFamide response, an action which was almost fully reversible. 0Na^+ saline application is marked by the bar.

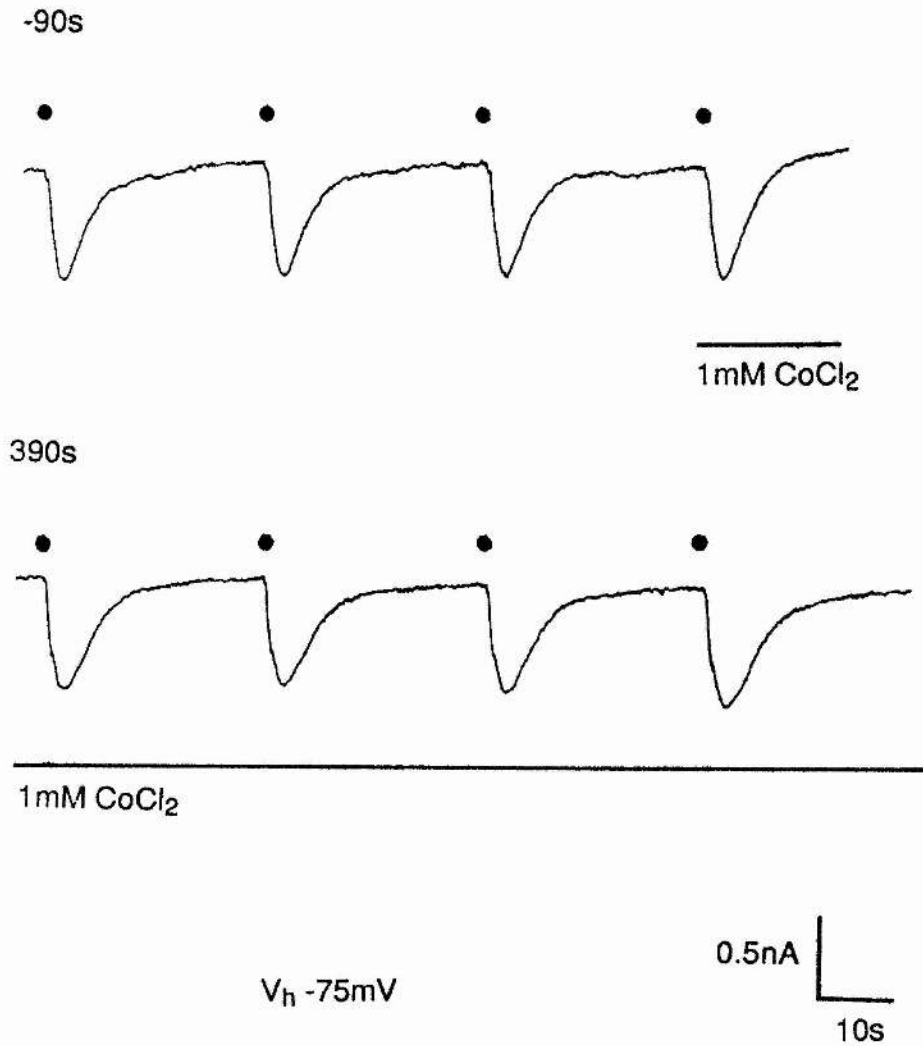


Figure 3.34

An experiment showing that normal saline containing 1mM CoCl₂ had no effect on the amplitude of the FMRamide response in a C2 neurone voltage clamped at -75mV. The administration of CoCl₂ is marked by the lines underneath the records. The time marked above each record refers to the first FMRamide response in that record relative to start of CoCl₂ application (time zero). FMRamide was applied at the points marked by the black dots.

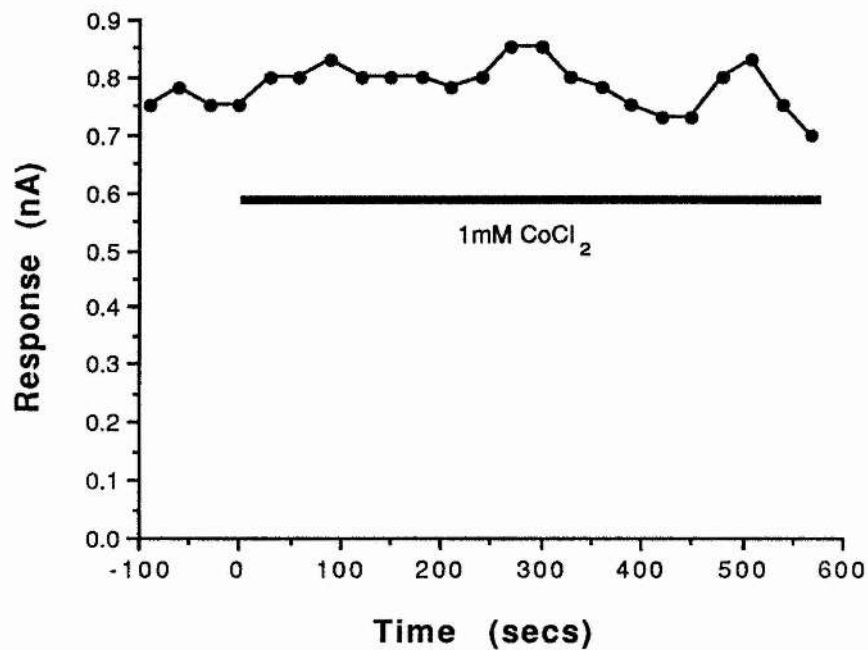


Figure 3.35

Data showing that 1mM CoCl₂ application to a C2 neurone held at -75mV had no effect on the amplitude of the FMRFamide response. The application of CoCl₂ is marked by the bar.

3.12.3 TETRODOTOXIN (TTX)

Since it had been established that Na^+ was the main charge carrier in the FMRFamide inward current response, the next stage was to determine whether the response could be blocked by drugs which are known to block Na^+ conductances.

TTX was used because its action is highly specific. When working with squid axons, Moore and coworkers in 1967 showed that TTX blocked the voltage sensitive Na^+ conductance selectively. For TTX to have any effect it must be applied outside the cell membrane.

Exposure of a C2 neurone to $50\mu\text{M}$ TTX did not reduce the amplitude of the FMRFamide response for 1200 seconds (20 minutes) (Figure 3.36, $n=3$). The data are represented in graphical form in Figure 3.37.

3.12.4 LIGNOCAINE

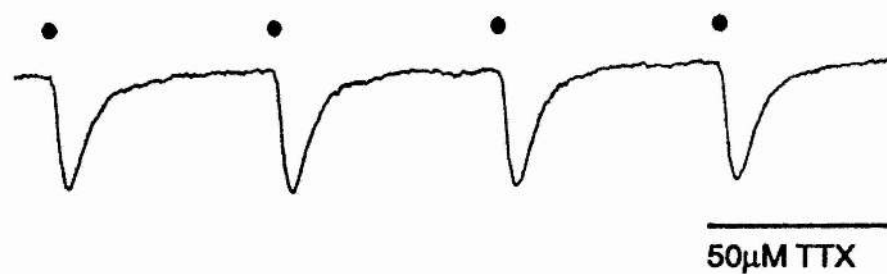
When local anaesthetics such as lignocaine are applied to nerves they increase the threshold for electrical stimulation, slow the rate of rise of the action potential and slow the rate of conduction. It is known that these drugs prevent the transient increase in Na^+ permeability that is essential for generating the rising phase of the spike potential in response to a depolarizing current (Covino, 1971; Lechat, 1971).

Application of 1mM lignocaine to a C2 neurone voltage clamped at -75mV had no effect on the amplitude

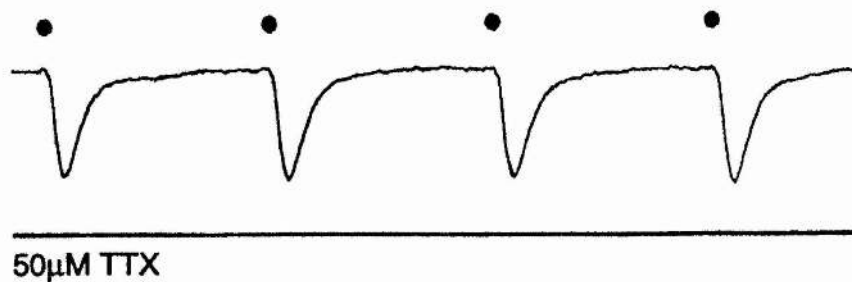
Figure 3.36

When 50 μ M TTX was applied to a C2 neurone voltage clamped at -75mV, there was no effect on the amplitude of the FMRFamide response. The time shown above each record refers to the first FMRFamide response in that record relative to the start of TTX application (time zero). The lines underneath the records show where TTX was applied. FMRFamide was pulsed onto the neurone at the points marked by the black dots.

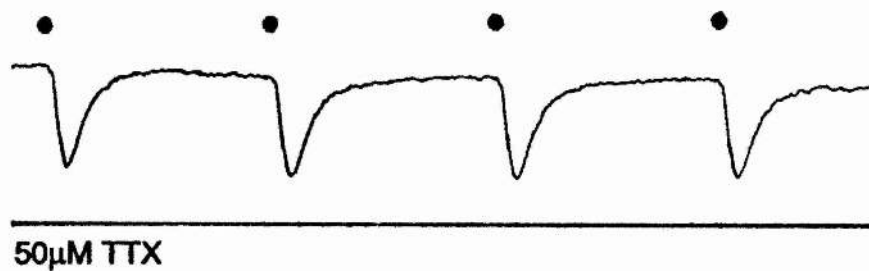
-90s



630s



1110s



0.5nA
10s

V_h -75mV

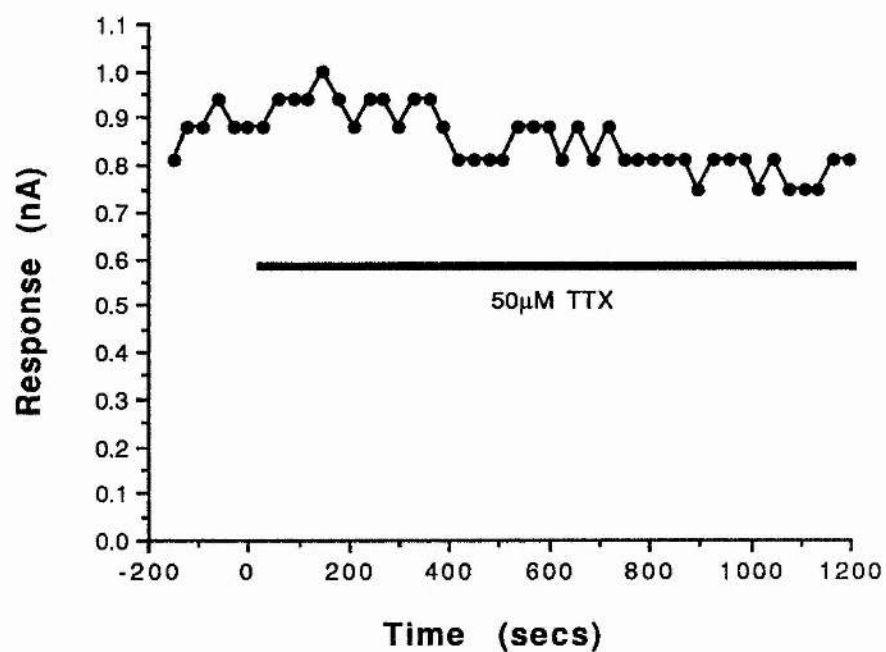


Figure 3.37

Data showing the lack of effect of 50µM TTX on the amplitude of the FMRFamide response of the C2 neurone voltage clamped at -75mV. Application of TTX is marked by the bar.

of the FMRFamide response (Figure 3.38) over a period of 810 seconds (13.5 minutes) as is shown in Figure 3.39 (n=2).

3.12.5 AMILORIDE

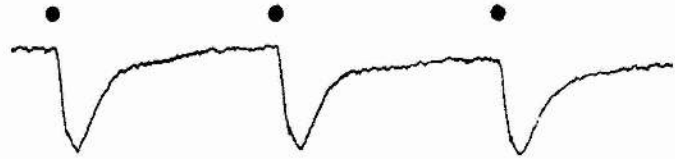
Amiloride is a specific and very potent inhibitor of Na^+ transport in a wide variety of epithelial and cellular transport systems throughout the animal kingdom (Benos, 1982). It is a drug which acts rapidly and reversibly and is used clinically as a K^+ sparing diuretic (Benos, 1982).

Amiloride was applied to the C2 neurone to see if it would inhibit the FMRFamide induced, fast Na^+ response. The experimental records in Figure 3.40 show that $100\mu\text{M}$ amiloride rapidly (within 60 seconds) almost completely blocked the FMRFamide response (n=5). This effect of amiloride was almost fully reversible as can be seen when amiloride was no longer present. The data from the experiment are illustrated in Figure 3.41. Of all the drugs tested, amiloride gave the most effective and reversible block.

3.13 EFFECT OF CYCLIC AMP ON THE FMRFamide RESPONSE OF THE C2 NEURONE

In a recent study by Song and Huang (1990), the modulation of glycine receptor channels by cyclic AMP dependent protein kinase A in neurones isolated from the spinal trigeminal nucleus of the rat was examined.

-90s



630s



1mM lignocaine

$V_h -75\text{mV}$

0.5nA

10s

Figure 3.38

The application of 1mM lignocaine to a C2 neurone voltage clamped at -75mV had no effect on the amplitude of the FMRamide response. Lignocaine application is marked by the line underneath the lower record. The time shown above each record refers to the first FMRamide response in that record relative to the start of lignocaine application (time zero). FMRamide was pulsed onto the neurone at the points marked by the black dots.

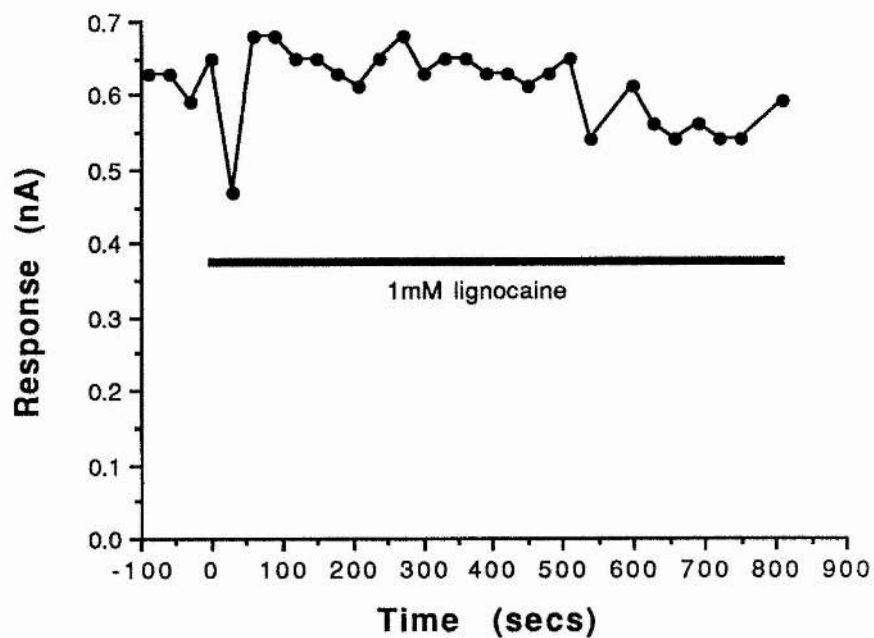


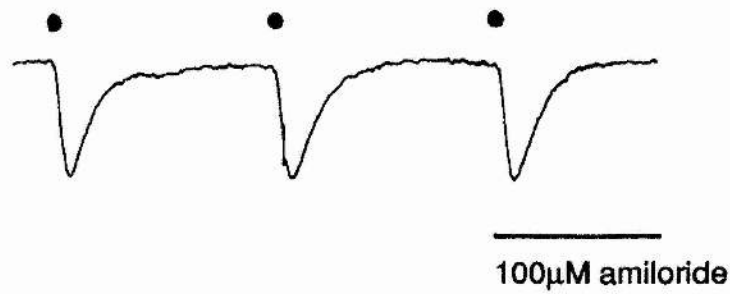
Figure 3.39

Data showing the lack of effect of 1mM lignocaine on the amplitude of the FMRFamide response of a C2 neurone which was voltage clamped at -75mV. The application of lignocaine is marked by the bar.

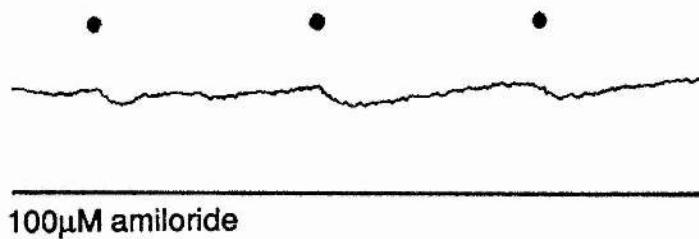
Figure 3.40

Amiloride at a concentration of $100\mu\text{M}$ reversibly reduced the amplitude of the FMRFamide response of a C2 neurone voltage clamped at -75mV . Amiloride application is marked by the lines underneath the upper and middle records. The time shown above each record refers to the first FMRFamide response in that record relative to the start of amiloride application (time zero). FMRFamide was pulsed onto the neurone at the points marked by the black dots.

-60s



60s



510s



Vh -75mV

0.5nA
10s

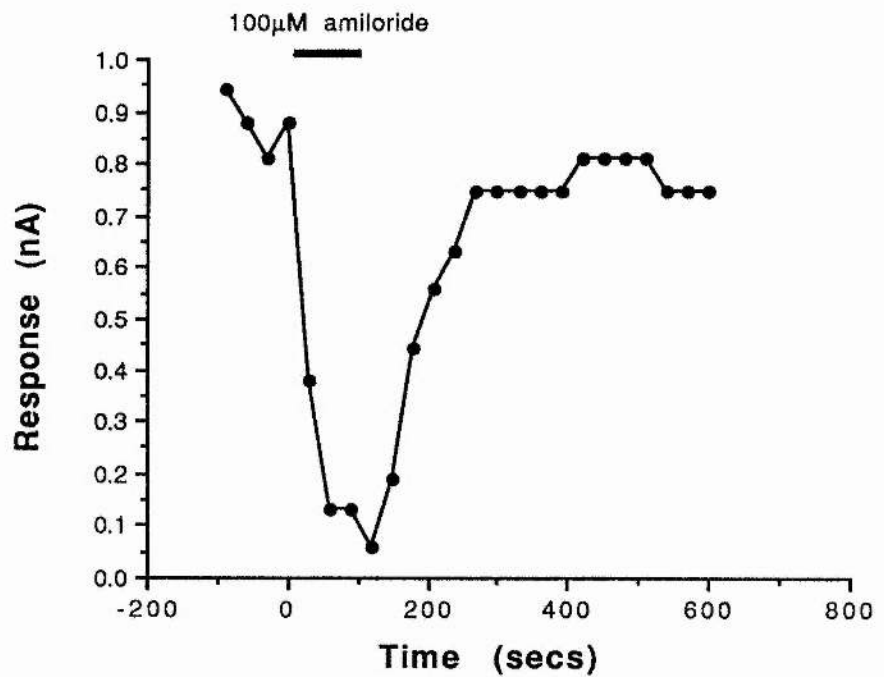


Figure 3.41

Data showing that 100µM amiloride application reversibly reduced the amplitude of the FMRFamide response of a C2 neurone voltage clamped at -75mV. Amiloride application is marked by the bar.

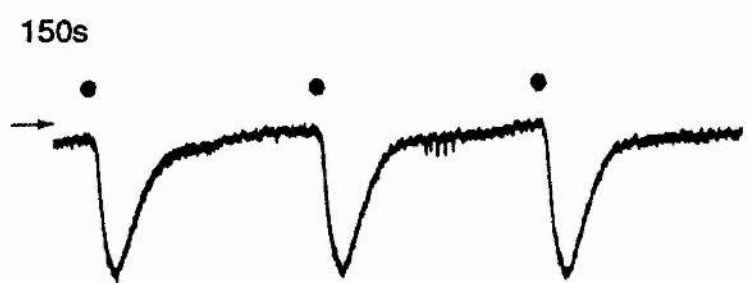
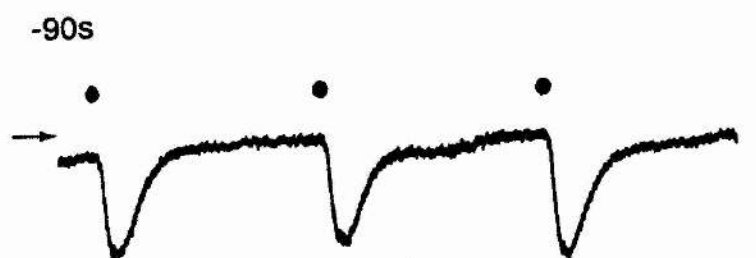
They found that activation of cyclic AMP dependent protein kinase dramatically increased the glycine induced Cl^- currents by increasing the probability of the channel openings. A G_s protein was also involved in the modulation. It was suggested that glycine receptors may belong to a superfamily of chemically gated channel proteins of which the GABA_A and nicotinic ACh receptors belong. Cottrell and coworkers (1990) have shown that the FMRFamide induced fast Na^+ response of the C2 neurone is due to the opening of a ligand gated channel. Therefore it was important to determine if this channel was also modulated by cyclic AMP and protein phosphorylation. Membrane soluble analogues of cyclic AMP were used in the experiments described below.

3.13.1 DIBUTYRYL CYCLIC AMP

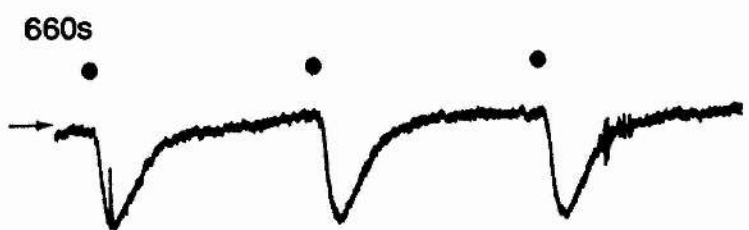
Dibutyryl cyclic AMP was the first analogue to be used. When this substance at a concentration of 1mM was applied to a C2 neurone, there was a rapid (within 150 seconds) increase in amplitude of the response ($n=5$) and a slight inward movement of the baseline current level. This can be seen by comparing the upper and middle records of Figure 3.42. When dibutyryl cyclic AMP was washed away (lower record Figure 3.42), the FMRFamide responses returned to their original amplitude and the baseline membrane current also returned to its original level. The data from the experiment are plotted in Figure 3.43. The inward

Figure 3.42

The application of 1mM dibutyryl cyclic AMP to a C2 neurone voltage clamped at -45mV reversibly potentiated the amplitude of the FMRFamide response. It also induced a very small inward baseline current. Dibutyryl cyclic AMP application is marked by the line underneath the middle record. The time marked above each record refers to the first FMRFamide response in that record relative to the start of dibutyryl cyclic AMP application (time zero). The arrows to the left of the records show the level of the baseline current at time zero. FMRFamide was pulsed onto the neurone at the points marked by the black dots.



1mM dibutyryl. cAMP



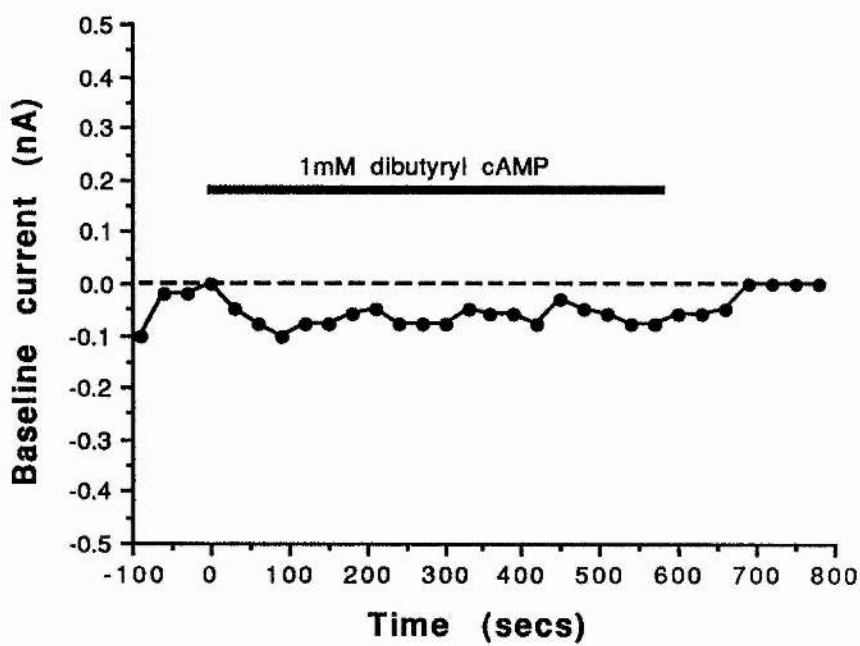
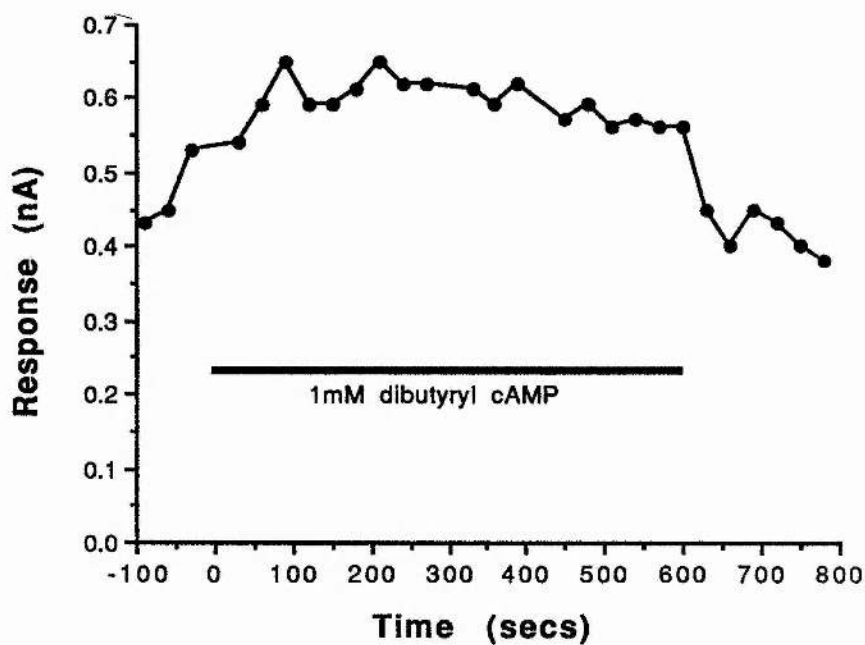
V_h -45mV

0.5nA

10s

Figure 3.43

Data showing that the application of 1mM dibutyryl cyclic AMP to a C2 neurone voltage clamped at -45mV reversibly potentiated the amplitude of the FMRFamide response (upper graph). It also produced a small reversible inward current (lower graph). Dibutyryl cyclic AMP application is shown by the bar on each graph. The level of the baseline current at time zero is marked by the dashed line on the lower graph.



current induced by dibutyryl cyclic AMP was not investigated further.

3.13.2 8-(-4) CPT CYCLIC AMP

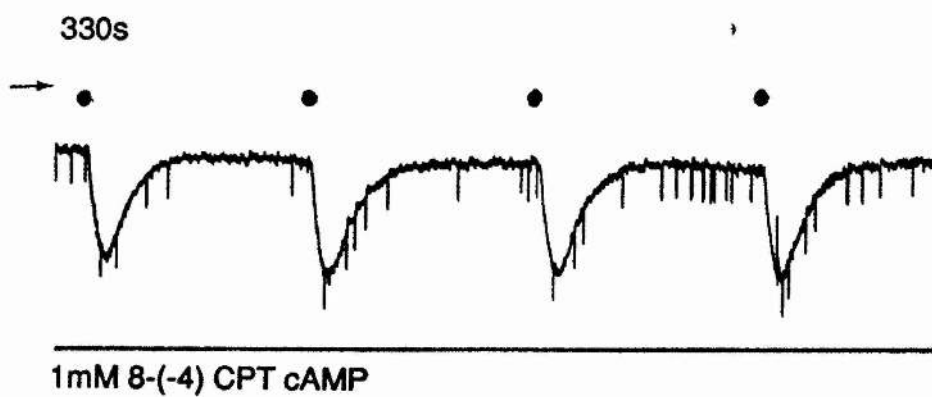
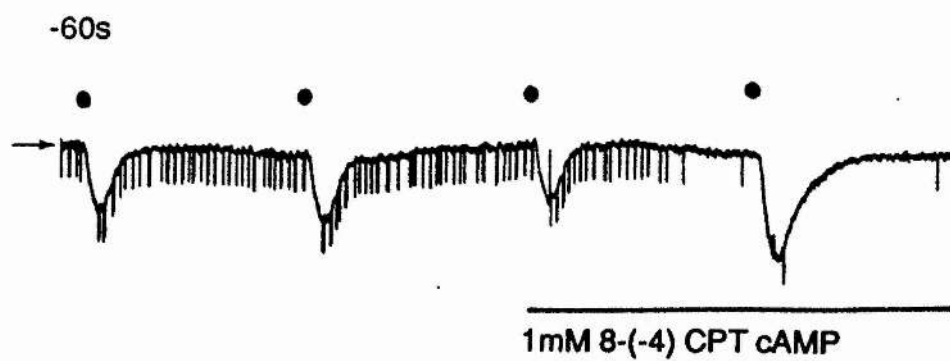
When the more potent membrane soluble analogue of cyclic AMP, 8-(-4) CPT cyclic AMP was used at a concentration of 1mM, the rapid increase in the amplitude of the FMRFamide response was even more pronounced (upper and middle records Figure 3.44). The analogue also produced an inward current which was larger than the dibutyryl cyclic AMP induced inward current. When the 8-(-4) CPT cyclic AMP was washed from the preparation (lower record Figure 3.44), the amplitude of the FMRFamide responses and the baseline membrane current returned to their original levels. The data from the experiment are plotted in Figure 3.45.

3.13.3 CYCLIC AMP

When using membrane soluble analogues of cyclic AMP at high concentrations, it was important to determine if the increase in the FMRFamide response might be due to some external, non-specific action of these substances. To test this possibility, cyclic AMP itself, which is not membrane soluble, was applied at a 1mM concentration to the C2 neurone. The data of an experiment are shown in Figure 3.46. Cyclic AMP had no significant effect on the amplitude of the FMRFamide response or the baseline membrane current. Therefore,

Figure 3.44

When 1mM 8-(-4) CPT cyclic AMP was applied to a C2 neurone voltage clamped at -45mV, the amplitude of the FMRFamide response was reversibly potentiated. An almost fully reversible inward baseline current was also induced. 8-(-4) CPT cyclic AMP application is marked by the lines underneath the upper and middle records. The time marked above each record refers to the first FMRFamide response in that record relative to the start of 8-(-4) CPT cyclic AMP application (time zero). The arrows to the left of the records show what was the position of the baseline current level at time zero. FMRFamide was pulsed onto the neurone at the points marked by the black dots. The rapid inward current deflections represent unclamped axon spikes.



$V_h -45\text{mV}$

1nA
10s

Figure 3.45

Data showing 1mM 8-(-4) CPT cyclic AMP reversibly potentiated the amplitude of the FMRFamide response of a C2 neurone which was voltage clamped at -45mV (upper graph). An almost fully reversible inward current was also induced (lower graph). 8-(-4) CPT cyclic AMP application is shown by the bar on each graph. The level of the baseline current at time zero is marked by the dashed line on the lower graph.

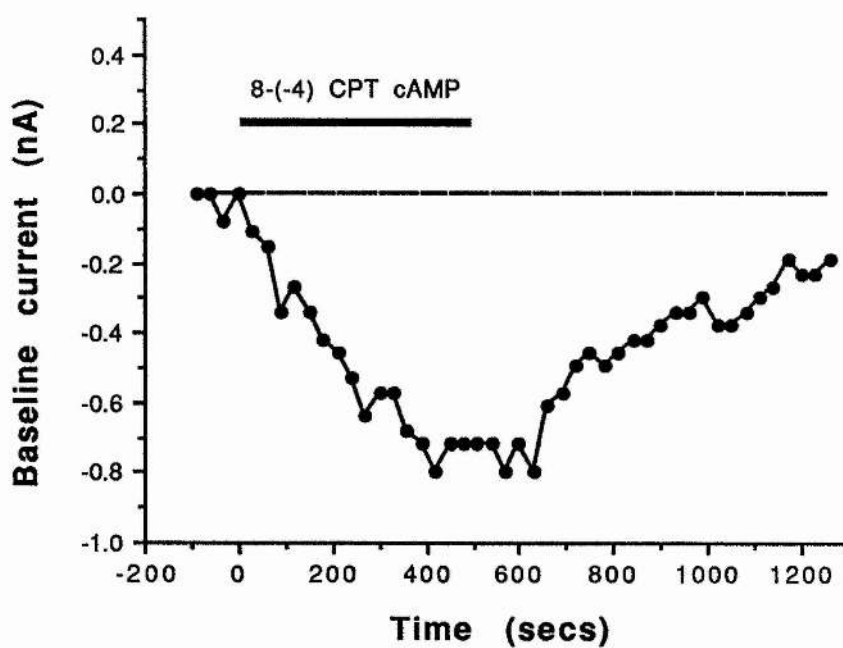
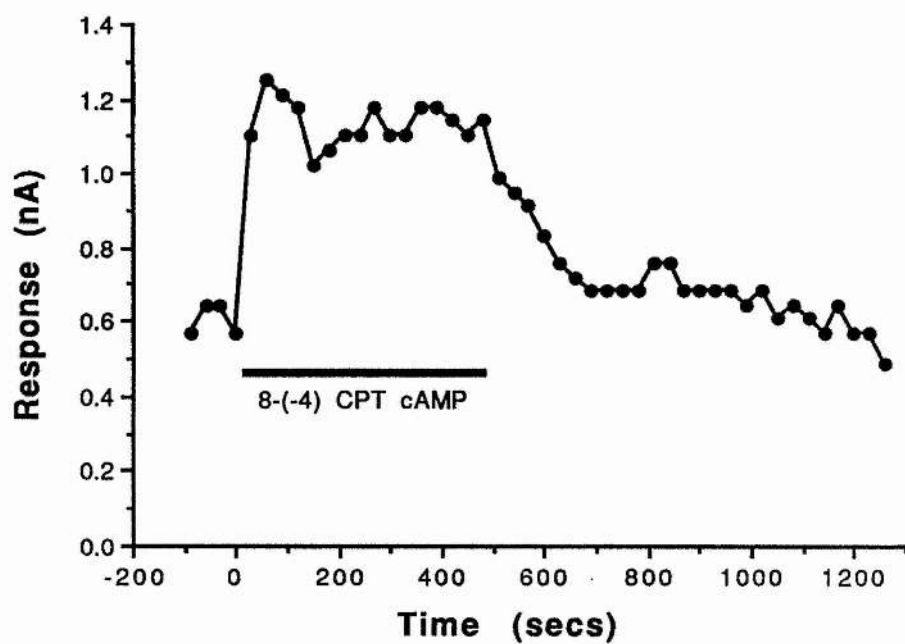
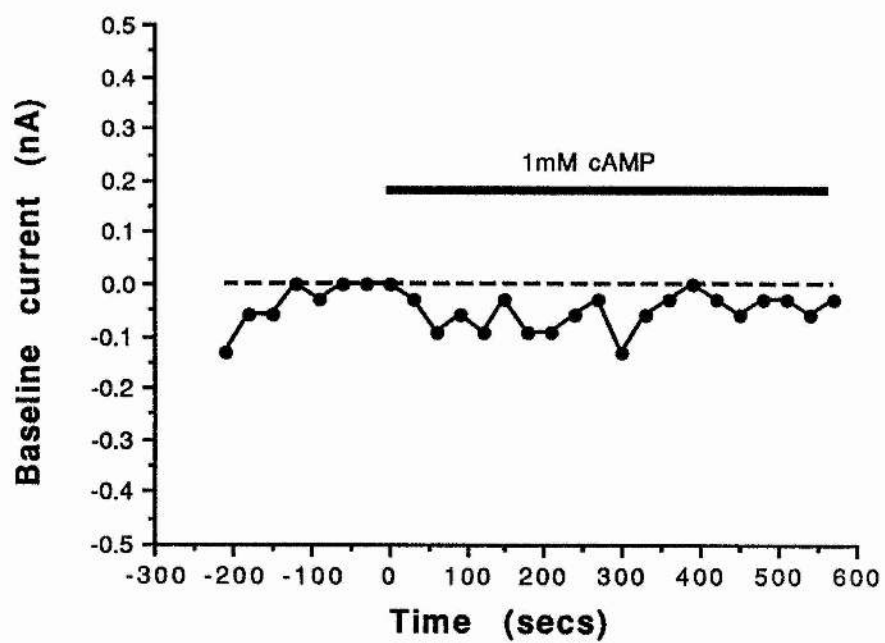
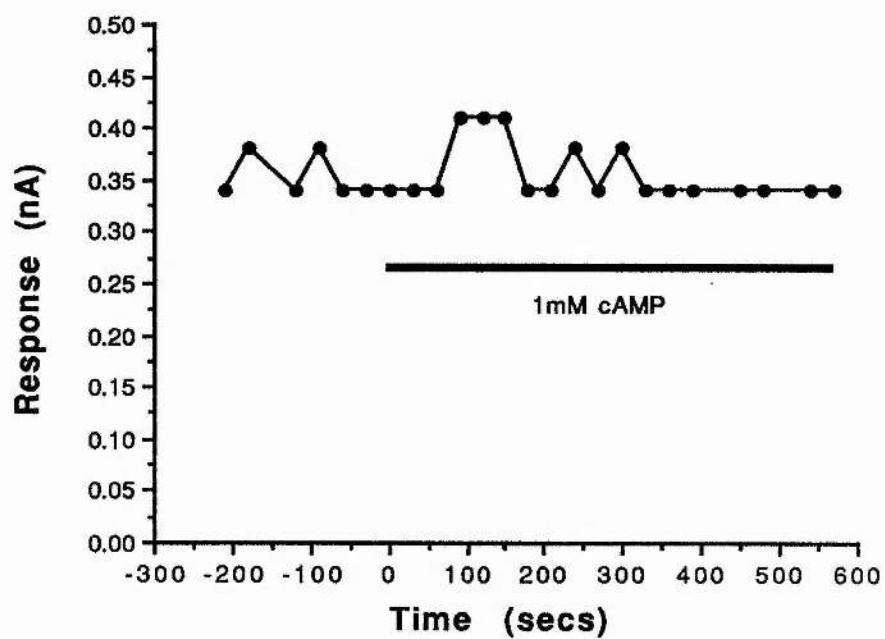


Figure 3.46

These data show that 1mM cyclic AMP application to a C2 neurone voltage clamped at -45mV had no effect on the amplitude of the FMRFamide response (upper graph) or the baseline current level (lower graph). The bar on each graph represents the application of cyclic AMP. The level of the baseline current at time zero is marked by the dashed line on the lower graph.



it appears that the effects of the membrane soluble analogues were due to intracellular action rather than an extracellular action.

3.13.3 IBMX

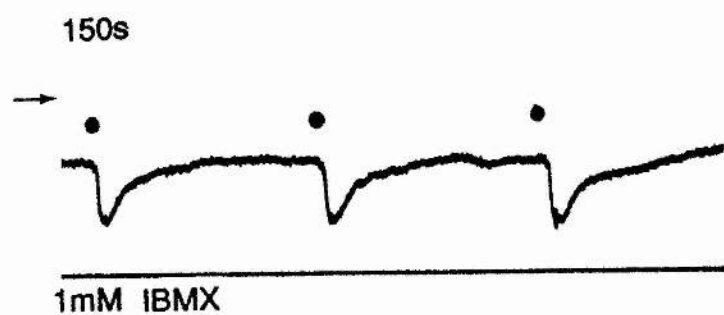
The phosphodiesterase inhibitor IBMX was applied to the C2 neurone. Since IBMX inhibits the breakdown of cyclic nucleotides, this was another means of raising the intracellular cyclic AMP levels. As was expected, exposure of the C2 to 1mM IBMX (n=3) led to an increase in amplitude of the FMRFamide response and the production of an inward current (see Figures 3.47 and 3.48). This was a mimic of the membrane soluble cyclic AMP analogue effects.

3.13.4 8-(-4) CPT CYCLIC AMP AND OKADAIC ACID

With the potentiation of FMRFamide response by the membrane soluble analogues of cyclic AMP having been established, it was important to determine if this occurred by increased protein phosphorylation due to cyclic AMP dependent protein kinase A activation. This was approached by applying the protein phosphatase 1 and 2A inhibitor, okadaic acid along with 8-(-4) CPT cyclic AMP to the C2 neurone. The idea was that if the potentiation of the FMRFamide response induced by 8-(-4) CPT cyclic AMP was due ultimately to increased protein phosphorylation, okadaic acid at a high

Figure 3.47

The application of 1mM IBMX to a C2 neurone voltage clamped at -73mV reversibly potentiated the amplitude of the FMRFamide response. It also induced a reversible inward current. IBMX application is shown by the line underneath the middle record. The time marked above each record refers to the first FMRFamide response in that record relative to the start of IBMX application (time zero). The arrows on the left of the records indicate the original baseline current level at time zero. FMRFamide was pulsed onto the neurone at points marked by the black dots.

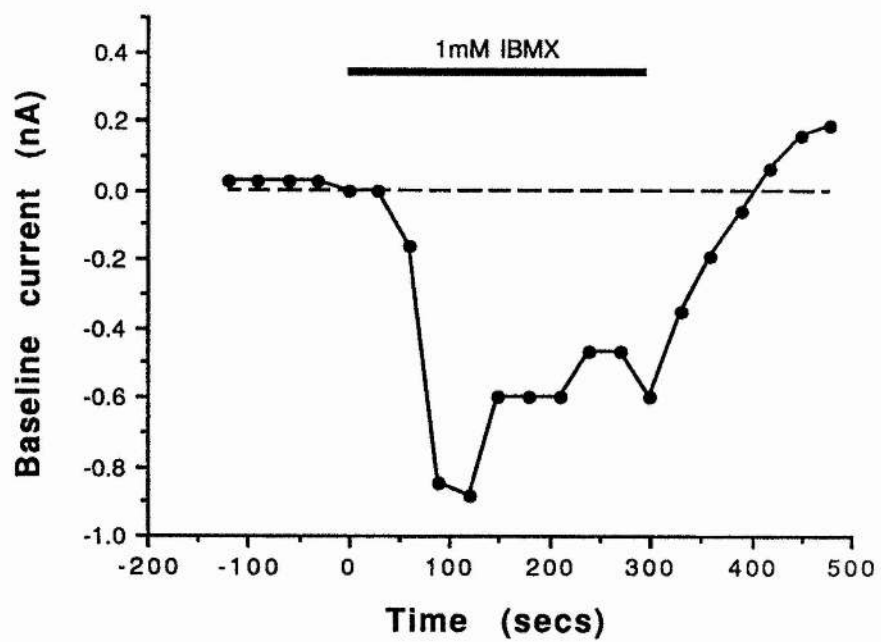
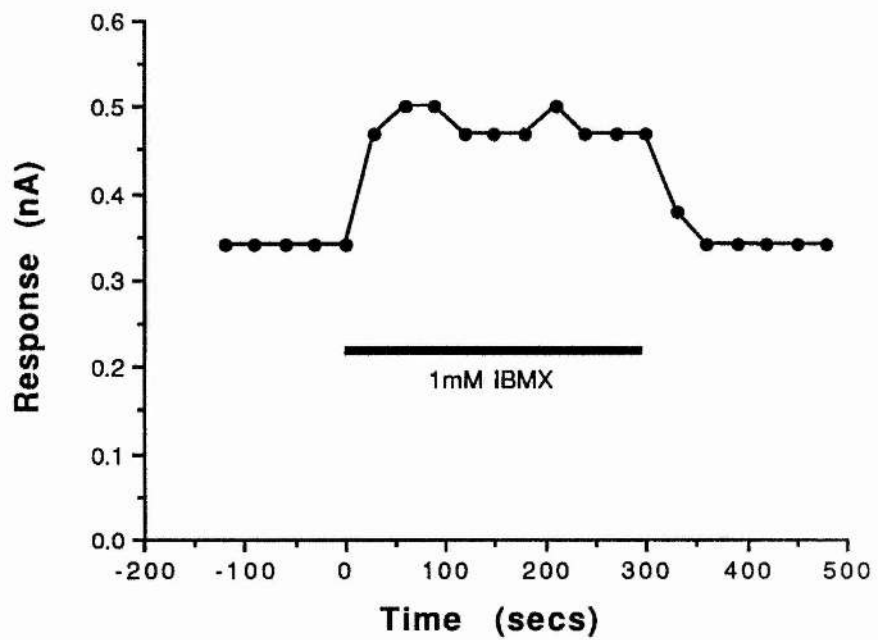


V_h -73mV

0.5nA
10s

Figure 3.48

Data showing that 1mM IBMX application to a C2 neurone voltage clamped at -73mV caused the reversible potentiation of the amplitude of the FMRFamide response (upper graph) and also produced a reversible inward baseline current (lower graph). IBMX application is marked by the bar on each graph. The original level of the baseline current at time zero is indicated on the upper graph by the dashed line.

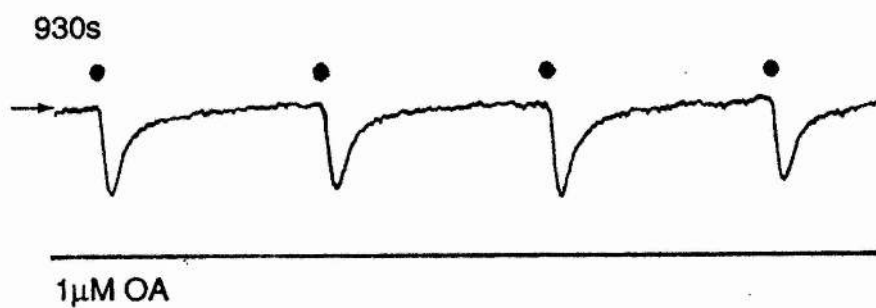
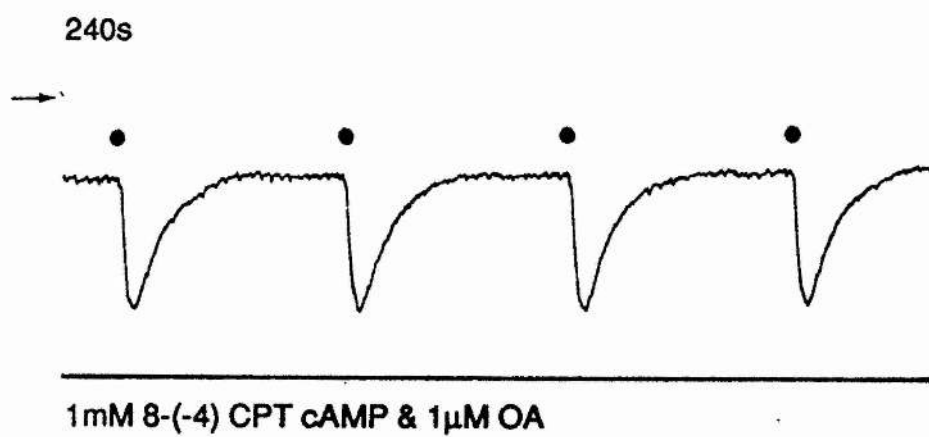
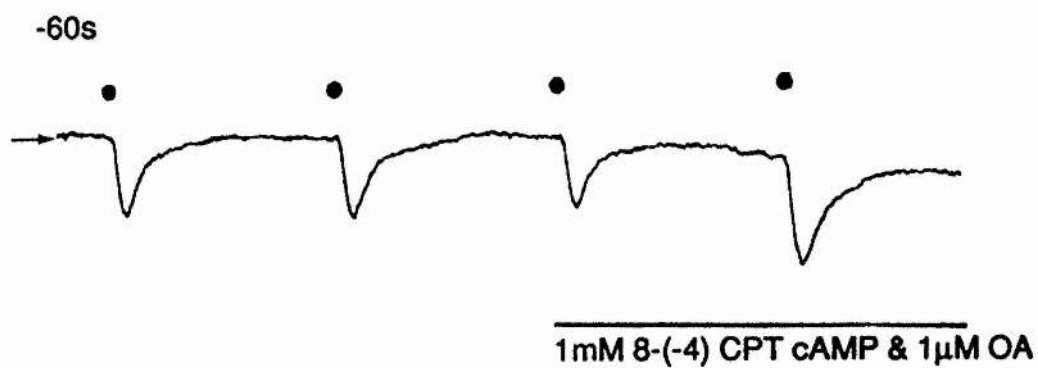


concentration ($1\mu\text{M}$) would prevent protein dephosphorylation and hence maintain the potentiation even in the absence of 8-(-4) CPT cyclic AMP.

Figure 3.49 shows that when 1mM 8-(-4) CPT cyclic AMP and $1\mu\text{M}$ okadaic acid were applied together to a C2 neurone, a familiar potentiation of the amplitude of the FMRFamide response was produced. However, when the neurone was then washed with $1\mu\text{M}$ okadaic acid only, the amplitude of the FMRFamide response reduced back towards control levels ($n=3$). The data obtained in the experiment are presented graphically in Figure 3.50.

Figure 3.49

The application of a combination of 1mM 8-(-4) CPT cyclic AMP & 1 μ M okadaic acid potentiated the amplitude of the FMRFamide response of a voltage clamped C2 neurone (V_h -75mV). It also induced an inward movement of the baseline current level. A following wash with 1 μ M okadaic acid reversed the two effects detailed above. 8-(-4) CPT cyclic AMP & okadaic acid application and okadaic acid alone are marked by the lines underneath the records. The time marked above each record refers to the first FMRFamide response in that record relative to the start of the 8-(-4) CPT cyclic AMP/okadaic acid application (time zero). The arrows on the left of the records indicate the original baseline current level at time zero. FMRFamide was pulsed onto the neurone at the points marked by the black dots.

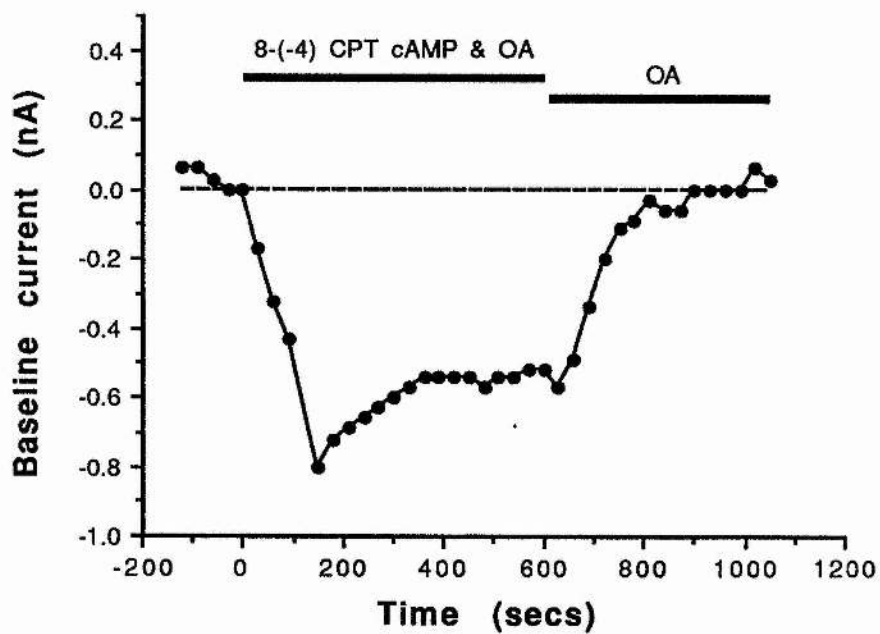
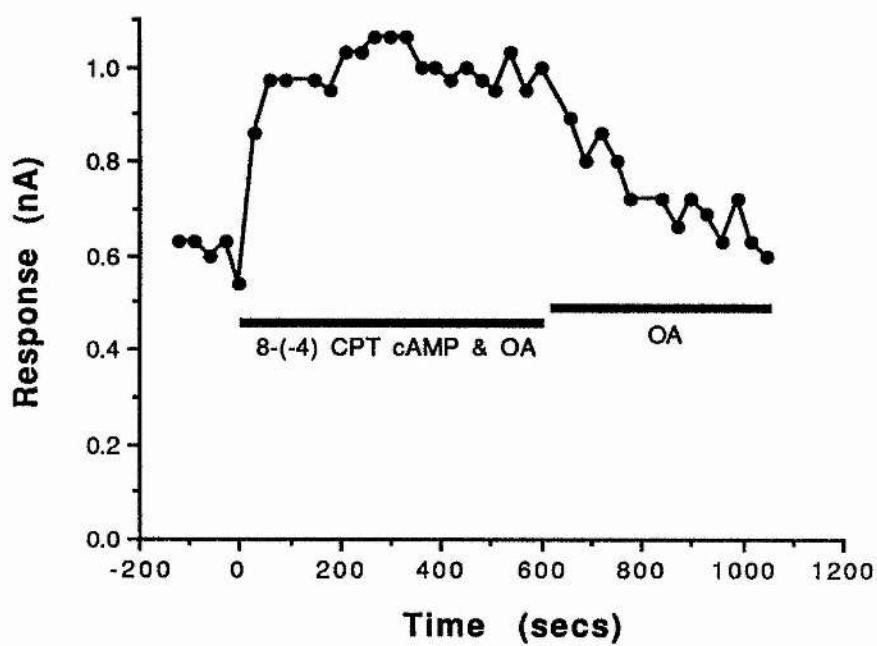


V_h -75mV

0.5nA
10s

Figure 3.50

Data showing that the combined application of 1mM 8-(-4) CPT cyclic AMP & 1 μ M okadaic acid produced a potentiation of the amplitude of the FMRFamide response in a C2 neurone voltage clamped at -75mV (upper graph). This combination also induced an inward current (lower graph). Both effects were reversed on washing with 1 μ M okadaic acid. The application of the 8-(-4) CPT cyclic AMP & 1 μ M okadaic acid mixture and 1 μ M okadaic acid alone are marked by the bars on each graph. The original position of the baseline current level at time zero is indicated on the lower graph by the dashed line.



Chapter 4

Discussion

4.1 GENERAL PROPERTIES OF THE FMRFamide RESPONSES

The purpose of the studies detailed in this thesis has been to examine two different effects of FMRFamide on identified molluscan neurones. The application of FMRFamide to the cell bodies of these neurones probably stimulates extra-synaptic receptors, since molluscan synapses are usually axo-axonic. Not only does FMRFamide induce currents carried by two separate cations, one K^+ and the other Na^+ , the profiles of the responses are also quite different. The K^+ current induced by FMRFamide in the C1 neurone is slow (7 seconds between application and maximal response) whilst the Na^+ current is relatively fast (2.5 seconds between application and maximal response). Such differences illustrate the diversity of action of FMRFamide related peptides on nervous tissue. The FMRFamide related peptides may act as neurotransmitters and also have a neurohormonal role.

4.2 THE SLOW K^+ CURRENT INDUCED BY FMRFamide IN THE C1 NEURONE

Previous studies have shown conclusively that the hyperpolarizing current induced by FMRFamide in the C1 neurone of Helix aspersa is carried by K^+ (Cottrell et al, 1984). The reversal potential obtained from the current response/voltage relationship (Figure 3.2) supports this finding. The reversal potential recorded was -70mV which is close to that predicted by the

Nernst equation (-75mV) for a conductance carried by K^+ alone. The intracellular concentration of K^+ was assumed to be 98mM (Alvarez-Leefmans and Gamino, 1982). One of the main questions addressed in this thesis is, what is the mechanism by which FMRFamide opens the K^+ channels? The fact that the response is slow implies that there is a considerable delay between FMRFamide receptor activation and channel opening. This suggests that activation of an intracellular second messenger system may underlie the FMRFamide response.

4.2.1 G PROTEIN INVOLVEMENT

G proteins are proteins housed in cell membranes which mediate signal transduction by interacting with receptor proteins. G proteins are structurally heterogeneous. They are heterotrimers made up of α , β and γ subunits. The β and γ subunits are closely associated with each other. This $\beta\gamma$ complex is thought to anchor the G protein to the plasma membrane (Bourne, 1986).

When G proteins are inactive, GDP is bound to the catalytic site of GTPase localized in the α subunit. The conformational change of a receptor induced by agonist binding influences the coupling G protein to release the bound GDP. Intracellular GTP then binds to the same site where it is hydrolysed by the GTPase. While this process of hydrolysis is taking place, the α subunit dissociates from the $\beta\gamma$ subunits. The α subunit reassociates with the $\beta\gamma$ subunits to form a more stable

conformation when GDP reoccupies the GTPase site. The non-hydrolysable GTP analogue, GTP γ S, is therefore able to irreversibly activate G proteins.

When GTP γ S was injected into the cell body of the C1 neurone, two clear effects were observed. Firstly, GTP γ S induced an outward current, which increased as a function of time. This outward current was due to an increase in membrane conductance. Secondly, the FMRFamide responses were reduced as the outward current associated with GTP γ S took effect. In other words, the responses to FMRFamide were occluded by the outward current. At the end of the experiments the outward current associated with GTP γ S was almost equal to the amplitude of the control FMRFamide responses. The responses to FMRFamide obtained prior to injection of GTP γ S in the experiment shown in Figure 3.4 were large (2.4nA) and close to maximum. The final level of the baseline current may have indicated a complete open state of almost all K⁺ channels, coupled with the total number of FMRFamide receptors in the neuronal cell body. This would have resulted in the occlusion of the FMRFamide responses. The effects of GTP γ S provide good evidence that G protein activation is required for the slow FMRFamide response in the C1 neurone.

The next stage was to examine what type of G protein was involved. Three broad groups of G proteins are recognised. G_s, G proteins which stimulate adenylate cyclase, G_i, G proteins which inhibit adenylate cyclase and G_o, G proteins which do not influence adenylate cyclase (Birnbaumer *et al*, 1990;

Taylor, 1990). The bacterial toxin pertussis toxin (PTX) has a protomer subunit which has an adenosine diphosphate (ADP) ribosyltransferase. This bacterial toxin can ADP-ribosylate α subunits of G_i and G_o G proteins. The ADP ribosylation by PTX occurs at the cysteine residue of these α -subunits and uncouples these G proteins from their receptors (Hsia et al 1984; Ui, 1984; Van Drop et al, 1984). PTX does not impair GTPase activity at the α subunit of G_i or G_o .

When PTX in its active form was injected into a C1 neurone, the FMRFamide responses were reduced. This evidence suggested that the G protein involved in the FMRFamide response was of the G_i or G_o type.

These results compare favourably with experiments performed by workers interested in the modulation of ion channels. Brezina (1988) using GTP γ S injections showed that FMRFamide activates an "S" like K^+ current by a mechanism involving a G protein in abdominal ganglion neurones of Aplysia. Sasaki and Sato (1987) and Sasaki et al, (1987) showed that K^+ channel openings induced by ACh, dopamine, histamine and FMRFamide were all produced via the activation of PTX sensitive G proteins.

4.2.2 CYCLIC AMP AND 5-HT

Membrane soluble analogues of cyclic AMP were used to mimic an intracellular rise in concentration of this second messenger. When 8-(-4) CPT cyclic AMP was applied to C1 neurones there were two clear effects.

Firstly, there was a steady reduction in the amplitude of FMRFamide responses until they were completely suppressed. Secondly, there was an inward movement of the baseline current level, which was associated with increased membrane conductance. There was certainly no mimic of the FMRFamide responses. This indicated that the receptor activated increase of cyclic AMP levels was not the mechanism underlying the FMRFamide response. Application of the phosphodiesterase inhibitor IBMX, which prevents the breakdown of both cyclic AMP and cyclic GMP also reduced the amplitude of the FMRFamide response and induced an inward current. The action of 5-HT appears to be important in relation to the above results. 5-HT induced an inward current and reduced the amplitude without complete suppression of the FMRFamide responses. Since the actions of 5-HT and raised cyclic AMP levels were similar it seemed reasonable to suggest that 5-HT, operating through the production of cyclic AMP, may be responsible for the observed effect. The likelihood of this being the case was enhanced by the results obtained from experiments where a mixture of IBMX and 5-HT were applied to the neurones. Compared to 5-HT application alone, 5-HT with IBMX produced a greater reduction in the amplitude of the FMRFamide response and the response took considerably longer to recover when the compounds were washed from the neurones. The inward currents induced by 5-HT and IBMX were of increased magnitude and took a greater time to recover.

Brezina et al (1987a) performed similar experiments on Aplysia abdominal ganglion neurones. The results reported agree with some of the observations made with C1 neurone. Direct injection of cyclic AMP and extracellular application of compounds which raise cyclic AMP levels, reduced the increase in K^+ conductance induced by FMRFamide in these Aplysia neurones. 5-HT application also reduced the FMRFamide responses, an effect which was enhanced in the presence of RO20-1724, a cyclic AMP phosphodiesterase inhibitor. The injection of cyclic AMP also induced an inward current which was Na^+ dependent. Intracellular injection of cyclic AMP has been shown to induce slow inward currents in many gastropod molluscs. It was therefore not surprising that treatments designed to increase intracellular cyclic AMP levels in the C1 neurone and Aplysia neurones induced inward currents due to increased membrane conductances. The inward current varies from one species to another. In neurones of Pleurobranchaea californica (Gillette and Green, 1987), Lymnaea stagnalis (McCrohan and Gillette, 1988) and Arachidoris montereyensis (Connor and Hockberger, 1984), it is carried purely by Na^+ . In Aplysia californica it is carried by Ca^{2+} (Pellmar, 1981) and in Limax maximus it is carried by both Na^+ and Ca^+ (Hockberger and Connor, 1984).

When Brezina et al (1987a) performed patch clamp studies on pleural ventrocaudal neurones, which responded in the same manner to FMRFamide as the abdominal ganglion neurones, the results differed from

those obtained with the C1 neurone. With the Aplysia neurones, 5-HT caused the closure of background K^+ channels in cell attached patches, while FMRFamide enhanced the activity of these channels. FMRFamide did not appear to recruit previously silent channels and patch studies confirmed the idea that the channels sensitive to 5-HT and FMRFamide were "S" type K^+ channels.

Patch clamp studies on the C1 neurone using cell attached patches showed that at patch potentials close to the resting membrane potential (V_p -45mV), there was no background channel activity. When FMRFamide was applied to these patches there was no sign of channel openings. Therefore, it is not clear whether the outward current induced by FMRFamide on the C1 neurone is due to the opening of genuine "S" type K^+ channels or to some other as yet undefined K^+ channel.

4.2.3 CYCLIC GMP

Application of the membrane soluble analogue of cyclic GMP, 8-bromo cyclic GMP to C1 neurones produced an inward current. The inward current was not studied in any detail. The amplitude of the FMRFamide responses was not influenced by the analogue. From this it was concluded that elevated levels of cyclic GMP could be ruled out as the second messenger which underlies the response.

The result obtained with cyclic GMP made it possible to clarify a point which arose from the

effects of IBMX. IBMX application reduced the amplitude of the FMRFamide responses, which could have been due an increase in the levels of cyclic AMP, cyclic GMP or both. However, since increased cyclic GMP levels had no effect on the amplitude of the FMRFamide response it became clear that the action of IBMX was due only to increased cyclic AMP levels.

Cyclic GMP has been shown to be an important second messenger underlying the effects of FMRFamide on the abdominal ganglion neurone R14 of Aplysia (Ichinose and McAdoo, 1988). In this neurone, FMRFamide induces a slow depolarizing response which is due to an inward current carried by Na^+ . Intracellular injections of cyclic GMP into the R14 neurone mimicked the FMRFamide response. IBMX enhanced the effects of cyclic GMP and FMRFamide.

Cyclic GMP has also been shown to be involved in the 5-HT induced increase in Ca^{2+} membrane conductance in identified ventral neurones of Helix aspersa (Paupardin-Tritsch et al, 1986a and b). Injection of cyclic GMP or extracellular application of zaprinast, a cyclic GMP phosphodiesterase inhibitor, both mimicked the effect of 5-HT. Also, injection of cyclic GMP dependent protein kinase potentiated the actions of 5-HT or zaprinast on the Ca^{2+} current. Therefore, raised cyclic GMP levels activating cyclic GMP dependent protein kinase is an important mechanism mediating the action of 5-HT on these neurones.

4.2.4 INOSITOL 1,4,5-TRISPHOSPHATE (IP₃)

Streb et al (1983) reported that the second messenger IP₃ released Ca²⁺ from an intracellular non-mitochondrial store. This Ca²⁺ store was found to be the endoplasmic reticulum (Streb et al, 1984; Prentki et al, 1984).

To examine if the production of IP₃ was involved in the FMRFamide response of the C1 neurone, IP₃ was injected directly into the cell body. IP₃ applied in this way had no effect on the baseline current level. Since there was no mimic of the FMRFamide response this apparently ruled out the involvement of IP₃.

A number of studies have shown that IP₃ is likely to play a role as an intracellular second messenger mediating the increase in conductance of the neuronal membrane activated by neurotransmitters. Amongst these, the peptide bradykinin which induces inositol lipid breakdown, has attracted a great deal of attention because electrophysiological studies of this receptor-mediated event demonstrated the involvement of phosphoinositide metabolites in neuronal transmission. It was found that intracellular injection of IP₃ or Ca²⁺ mimicked the transient hyperpolarization induced by bradykinin in neuroblastoma X glioma hybrid NG108-15 cells (Higashida and Braun, 1986). Oron and coworkers (1985) have shown that injection of IP₃ into a Xenopus oocyte mimicked a muscarinic depolarizing Cl⁻ current,

hence implicating IP_3 as the second messenger mediating the response.

IP_3 has also been shown to have important effects on molluscan neurones. Fink and coworkers (1985) demonstrated that intracellularly injected IP_3 hyperpolarized the petidergic bag cell neurones of Aplysia, and caused a reduction in the action potentials evoked by depolarizing current. Also, injection of IP_3 into identified abdominal ganglion neurones of Aplysia induced an outward, hyperpolarizing K^+ current (Sawada et al, 1987).

4.2.5 PROTEIN KINASE C

Protein kinase C activation in the C1 neurone was achieved by applying a tumour promoting phorbol ester, phorbol 12,13-dibutyrate. Protein kinase C activation did not mimic the FMRFamide response hence ruling out the possibility of its direct involvement in producing the response. However, application of the phorbol ester did induce a reversible inward current and an irreversible reduction of the amplitude of the FMRFamide response. The inward current was not studied in detail. It is possible however, that the observed inward current with the phorbol ester, is related to larger inward currents seen at more depolarized potentials (less than $-45mV$) when 5-HT induces closure of voltage dependent K^+ channels in the C1 neurone. This effect of 5-HT has been shown to be mediated by protein kinase C and protein phosphorylation

(Hill-Venning and Cottrell, 1988; Cohen et al, 1989). At potentials less negative than -45mV FMRFamide is also thought to close the same voltage dependent K^+ channels as 5-HT (Davies, 1986).

The reduction in the amplitude of the FMRFamide response indicated that protein kinase C activation may in some way modulate the response. This finding falls in line with some other studies. For example, in hippocampal pyramidal neurones adenosine, baclofen and 5-HT induce a K^+ current which causes hyperpolarization. Pertussis toxin inhibited the action of these agonists which suggested that they operated through the activation of a G_i or G_o protein. When phorbol esters were applied to these neurones the responses were blocked, which indicated that protein kinase C activation might be able to inhibit the G protein that couples the receptors to the ion channels (El-Fakahany et al, 1988). It had been previously been shown with platelets that the activation of protein kinase C leads to the phosphorylation and inactivation of a G_i protein (Katada et al, 1985).

Since it has been shown that the FMRFamide response in the C1 neurone is mediated through a G_i or G_o protein, it is possible that protein kinase C activation could phosphorylate the G protein and cause its inactivation. However, the reason for the irreversible nature of this effect when the phorbol ester has been washed away is unclear.

4.2.6 OCa²⁺ EXPERIMENTS

Experiments were performed to examine if Ca²⁺, either entering the neurone from the extracellular environment through Ca²⁺ channels or being released from intracellular stores, are required for the generation of the FMRFamide response. Ca²⁺ concentrations were reduced either by injecting the Ca²⁺ chelator EGTA into the cell body of the neurone, or by removing Ca²⁺ from the perfusing saline. Both of these approaches produced quite a large, prolonged inward current. The effect was reversible in the OCa²⁺ saline experiments upon washing with normal saline. The shift in background current in an inward direction may have been due to the closure of Ca²⁺ dependent K⁺ channels. Neither treatment however, altered the amplitude of the FMRFamide response on the C1 neurone. The FMRFamide response can therefore be considered as Ca²⁺ independent.

This result falls into line with that obtained from the injection of IP₃. IP₃ injection, which has the role of releasing Ca²⁺ from intracellular non-mitochondrial stores into the cytosol, had no direct involvement in the FMRFamide response. The result also confirms that obtained with protein kinase C activation, which showed that this enzyme is not directly involved in the FMRFamide response. Activation of protein kinase C depends upon the presence of Ca²⁺ (El-Fakahany et al, 1988). If the FMRFamide response

had depended on protein kinase C activation, the reduction in Ca^{2+} concentration would have been expected to reduce the FMRFamide response.

4.2.7 ARACHIDONIC ACID

The second messenger arachidonic acid and its metabolites have been shown to mediate the FMRFamide action of increasing "S" K^+ channel openings in sensory neurones of Aplysia. Application of arachidonic acid to these sensory neurones was shown to mimic the FMRFamide response (Piomelli et al, 1987). The effect of FMRFamide on these neurones has been shown to be mediated by two separate actions: a) In the presence of basal cyclic AMP levels the FMRFamide induced increase in "S" K^+ channel open probability (Belardetti et al, 1987) occurs through the activation of the 12-lipoxygenase arachidonic acid breakdown pathway. 12-HPETE was the active metabolite of this pathway and its application to sensory neurones mimicked the FMRFamide response (Piomelli et al, 1987). It has now been suggested that 12-HPETE requires further metabolism to cause the opening of the "S" K^+ channels (Piomelli et al, 1989). These actions of FMRFamide and arachidonic acid metabolites do not involve phosphorylation or dephosphorylation reactions as was described by Buttner et al (1989). They were able to show that 12-HPETE could increase the probability of "S" K^+ channel opening in cell free membrane patches in the absence of ATP and GTP: b) When cyclic AMP levels were elevated

FMRFamide was able to antagonize the actions of cyclic AMP by reopening "S" K^+ channels. It is thought that this effect of FMRFamide was also mediated by a lipooxygenase arachidonic acid metabolism pathway. This eventually leads to the dephosphorylation of the "S" K^+ channel (Sweatt et al, 1989). Given these actions of FMRFamide on the sensory neurones and their mediation of them by arachidonic acid and its metabolites, it was important to test if arachidonic acid was involved in the FMRFamide induced K^+ response of the C1 neurone.

When arachidonic acid was applied to the C1 neurone there was no mimic of the FMRFamide response or any effect on the response. Therefore, unlike the "S" K^+ channels of sensory neurones of Aplysia, FMRFamide does not appear to act through arachidonic acid or its metabolites on the C1 neurone.

4.2.8 PROTEIN PHOSPHORYLATION

To determine whether protein phosphorylation or dephosphorylation reactions are involved in the generation of the FMRFamide response, okadaic acid was used. This substance inhibits the protein dephosphorylating actions of protein phosphatases 1 and 2A. If these protein phosphatases were operable in the C1 neurone, their inhibition should produce an increase in the level of protein phosphorylation.

When okadaic acid was applied to the C1 neurones, the amplitude of the FMRFamide response was greatly reduced. Therefore protein phosphatases 1 and /or 2A do

appear to operate in the C1 neurone. The results suggest that higher protein phosphorylation levels lead to a smaller FMRFamide response. This result corresponds with those obtained with increased cyclic AMP levels and protein kinase C activation. Both treatments should have resulted in increased protein phosphorylation levels and, as was demonstrated, the amplitude of the FMRFamide response was reduced.

This result with okadaic acid is consistent with results reported by Sweatt et al (1989) in which Aplysia sensory neurones are demonstrated to respond to FMRFamide with increased open probability of "S" K^+ channels. The resting levels of protein phosphorylation were reduced in the presence of FMRFamide without acting on the level of cyclic AMP. It is also in agreement with work done by Ichinose and Byrne (1991), where intracellular application of okadaic acid reduced the amplitude of the FMRFamide induced outward K^+ current in Aplysia sensory neurones. They suggested that FMRFamide may exert its effect through the activation of protein phosphatases. Slow diffusion of the catalytic subunits of protein phosphatases 1 and 2A produced a gradual outward shift in baseline current and this was associated with an increase in membrane input conductance. Thus these protein phosphatases mimicked the FMRFamide response.

Biochemical evidence for activation of protein phosphatases by transmitters was reported in striatal neurones where stimulation of NMDA receptors activates

protein phosphatase 2B, leading to the dephosphorylation of DARPP-32 (Halpain et al, 1990).

FMRFamide may therefore activate protein phosphatases in the C1 neurone and hence reduce the levels of protein phosphorylation and opening of K^+ channels.

4.2.9 SINGLE CHANNEL RECORDINGS

Single channel recordings were obtained from C1 neurones by employing the technique of cell attached patches. With this system an externally applied agonist has no direct means of access to the patch of membrane which is under investigation. Any contact must be made through the production of an intracellular second messenger.

Unitary currents obtained from cell attached patches on the C1 neurone became more obvious at patch potentials more depolarized than -45mV . When the concentration of K^+ in the recording pipette was reduced from 5mM to 1mM , the unitary currents were larger and easier to see at each potential. The channel permeabilities were similar with 5mM or 1mM K^+ in the recording pipette. The permeability values obtained were $1.68 \times 10^{-13}\text{cm}^3\text{s}^{-1}$ with 5mM K^+ and $1.62 \times 10^{-13}\text{cm}^3\text{s}^{-1}$ with 1mM K^+ .

No unitary currents were observed from patches when FMRFamide was applied to the outside of the cell body membrane. One reason for this may be that FMRFamide does not open the channels via the production

of an intracellular second messenger. Another explanation could be that the FMRFamide receptors and/or ion channels opened up by FMRFamide are located in an inaccessible area for patch clamp recording. Such an area might be the axon hillock region of the neurone. One way to approach this problem would be to culture the neurones in spherical form (without axonal processes) and hope to see a redistribution of receptors or ion channels over the cell body which were originally located at the axon hillock.

4.3 CONCLUSION OF EXPERIMENTS ON THE C1 NEURONE

It has been shown that a pertussis toxin sensitive G_i or G_o protein mediates the FMRFamide response. Of the second messenger systems examined, namely cyclic AMP, cyclic GMP, arachidonic acid, IP_3 , Ca^{2+} and protein kinase C activation, none appeared to be directly involved in underlying the FMRFamide response. The exclusion of these second messenger systems were supported by the negative results obtained from the cell attached patch experiments.

However, some substances did modulate the FMRFamide response. The neurotransmitter 5-HT and cyclic AMP both reduce the response amplitude. It seemed that 5-HT may have had this effect through the production of cyclic AMP. Protein kinase C activation also reduced the FMRFamide response as did higher levels of protein phosphorylation.

Given the positive G protein results and negative direct effect of second messengers, the idea that the FMRFamide receptor may be coupled to the ion channel directly through the activation of a G protein is proposed. The first described and most thoroughly examined example of such a mechanism is the ACh activated inwardly rectifying K^+ channels in atrial cardiac myocytes (Soejima and Noma, 1984; Pfaffinger et al, 1985; Kurachi et al, 1986a and b; Yatani et al, 1987a and b). The next question is how does protein phosphorylation and the suppression effects of 5-HT, cyclic AMP and protein kinase C activation fit into this scheme? It is possible that 5-HT acting through raised cyclic AMP levels and protein kinase C activation by diacylglycerol (DAG) could cause the phosphorylation of the FMRFamide receptor, G protein or ion channel and somehow reduce the activity of this complex. If protein phosphatases which dephosphorylate these proteins were inhibited, then this increase in protein phosphorylation may also reduce the FMRFamide response.

To test the validity of this model, many more patch clamping experiments would be required. Taking a similar approach as those who worked on the K^+ channel opened by ACh would be useful with perhaps the first step to get cell attached patches and to determine if FMRFamide in the recording pipette would have any effect (Soejima and Noma, 1984). If the results proved positive, the next stage would be to obtain inside out patches and see if channel activity remained. If

channel activity was lost then addition of GTP to the inside face of the inside out patch might be expected to restore channel activity if a G protein was involved (Kurachi et al, 1986a). It would also be useful to check if this activity was abolished by pertussis toxin pretreatment of the neurones (Kurachi et al, 1986b). Finally, it could also be possible to apply a purified and activated G protein to the inside surface of the inside out patch and again determine whether channel activity reappeared (Yatani et al, 1987b). For a model of this system see Figure 4.1.

Another explanation of the results could be that FMRFamide activates protein phosphatase(s) to reduce protein phosphorylation levels and open channels as was suggested by Ichinose and Byrne (1991) on Aplysia sensory neurones. Protein phosphorylation levels could be enhanced by 5-HT acting through cyclic AMP and DAG activation of protein kinase C, which would lead to a reduction in amplitude of FMRFamide responses. A model of this system is shown in Figure 4.2.

4.4 THE FAST DEPOLARIZING FMRFamide RESPONSE OF THE C2 NEURONE

FMRFamide induces a fast depolarizing response in the C2, F2 and E13 neurones of Helix apersa. In a study performed by Cottrell et al (1990), experiments were made on excised outside out patches from C2 neurones. In some instances when FMRFamide was applied to these patches, unitary currents appeared. Since these patches

Figure 4.1

A schematic representation of a possible mechanism which underlies the slow K^+ hyperpolarizing response of FMRFamide in the C1 neurone. In this model, the activated FMRFamide receptor housed in the lipid bilayer of the membrane is linked to the K^+ channel through the activation of a pertussis toxin sensitive G_i or G_o protein. Protein phosphorylation on any of the proteins making up the receptor /G protein /ion channel complex may down regulate its activity.

5-HT acting on its membrane receptor probably activates adenylate cyclase through a G_s protein which leads to the elevation of cyclic AMP levels. Cyclic AMP activates cyclic AMP dependent protein kinase A which would increase protein phosphorylation levels. The activation of protein kinase C also results in increased protein phosphorylation and reduce the activity of the FMRFamide receptor /G protein /ion channel complex. Protein phosphatases 1 and /or 2A are able to dephosphorylate the proteins of the complex. Inhibition of these protein phosphatases by okadaic acid again leads to increased protein phosphorylation levels of the complex and a smaller FMRFamide response.

The abbreviations used in this model are:- R= receptor, Fa= FMRFamide, 5-HT= 5-hydroxytryptamine, G_s = G protein which stimulates adenylate cyclase, G_i = G protein which inhibits adenylate cyclase, G_o = G protein with some other function, += an activation of, or increased levels of, as appropriate, -= reduced levels of. The bilayer of the cell membrane is represented by the shaded circles and the area between them.

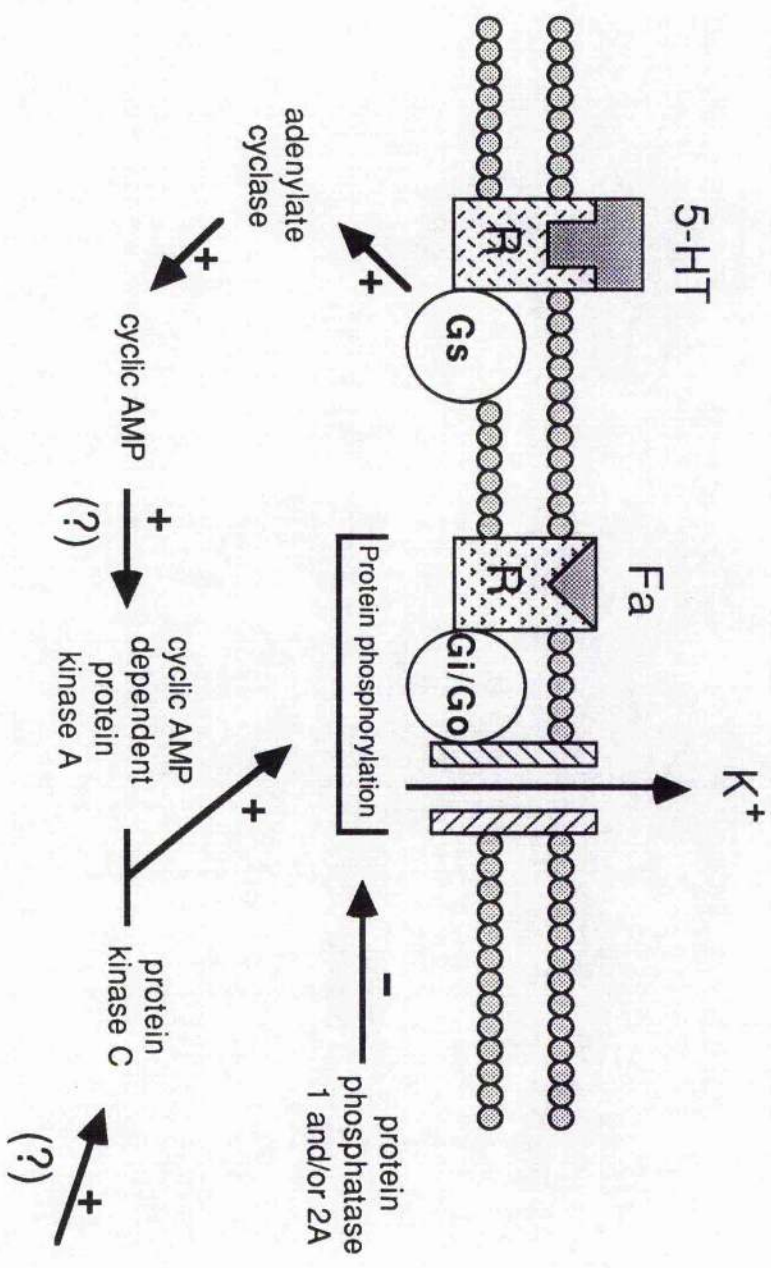
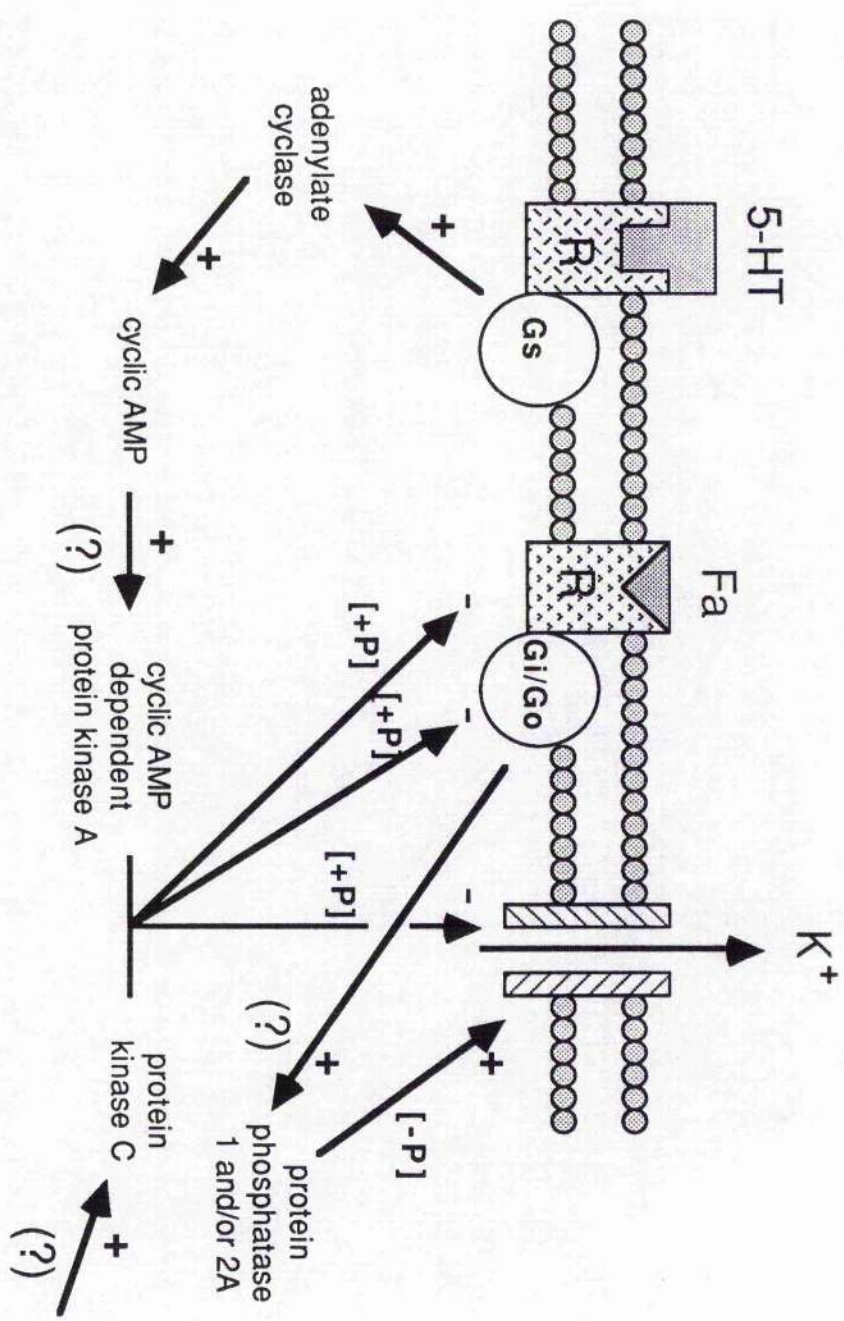


Figure 4.2

A schematic representation of another possible mechanism which underlies the slow hyperpolarizing K^+ response of FMRFamide in the C1 neurone. In this model, FMRFamide acting on its receptor activates a G_i or G_o protein which is linked by some intracellular mechanism to the activation of protein phosphatase 1 and /or 2A. Protein phosphorylation of the ion channel by these protein phosphatases leads to K^+ channel opening.

5-HT acting on its receptor to stimulate G_s and hence adenylate cyclase raises cyclic AMP levels. Cyclic AMP activates cyclic AMP dependent protein kinase A which can phosphorylate the FMRFamide receptor, G_i / G_o protein or the K^+ channel itself which causes the closure of the channel. The stimulation of protein kinase C may also lead to the phosphorylation of these proteins leading to the closure of the channel.

The abbreviations used in this model are:- R= receptor, Fa= FMRFamide, 5-HT= 5-hydroxytryptamine, G_s = G protein which inhibits adenylate cyclase, G_o = G protein with some other function, P= protein phosphorylation, += an activation or increased levels of, as appropriate, -= inactivation of or reduced levels of, as appropriate. The lipid bilayer of the cell membrane is represented by the shaded circles and the area between them.



were maintained in isolation from the C2 neurone for up to 20 minutes it was unlikely that a second messenger was directly underlying this response. Therefore, the only remaining explanation was that these were ligand gated channels opened by FMRFamide. This is most unusual since the effects of neuropeptides on neurones described to date have exclusively found that they operate through the generation of second messengers. Brezina et al (1988) have also reported that the fast FMRFamide induced Na^+ current is not mediated by the activation of a G protein.

Comparisons have been made between the partially characterized responses in the E13 and F2 neurones to FMRFamide (Cottrell et al, 1984; Cottrell et al, 1987; Cottrell et al, 1990) and the response in the C2 neurone. However, the response in the C2 neurone remained to be characterized.

4.4.1 DEPENDENCY OF THE RESPONSE ON Na^+ AND Ca^{2+}

To confirm that Na^+ was the charge carrier in the fast response, a modified saline containing ONa^+ was applied to the neurone. The ONa^+ saline diminished but did not completely abolish the response. The diminishing effect of ONa^+ saline was reversed when normal saline was washed over the neurones. These experiments showed that the FMRFamide response was dependent on Na^+ and suggest that Na^+ carries most of the FMRFamide induced current in the C2 neurone.

An effective and simple way to test whether Ca^{2+} ions are involved in the response is to apply CoCl_2 . CoCl_2 has been previously shown to block the Ca^{2+} current of molluscan neurones (Akaike *et al*, 1981). This approach did not directly deprive the neurones of Ca^{2+} in the intracellular or extracellular environment as does happen when either EGTA is injected into the neurones or applying OCa^{2+} saline. Therefore any other Ca^{2+} dependent processes not directly affected by Ca^{2+} entry through these channels would still be expected to function.

When C2 neurones were bathed in a normal saline, which contained 1mM CoCl_2 , there was no change in the amplitude of the FMRFamide response. These results indicate that the fast FMRFamide current is not carried by Ca^{2+} and that Ca^{2+} are not required in another way for the generation of the response.

4.5 SEARCH FOR A BLOCKER OF THE FAST FMRFamide RESPONSE

A number of substances known to block Na^+ channels and to antagonize receptors which lead to the opening of Na^+ channels were used.

4.5.1 TETRODOTOXIN (TTX)

TTX was the first substance tested on the C2 neurone. A long term exposure of the neurones to 50 μM TTX (20 minutes) had no effect on the amplitude of the

FMRamide responses. TTX is a substance which is known to selectively block voltage sensitive Na^+ currents (Moore et al, 1967). The FMRamide response of the C2 neurone is voltage independent. Therefore, it was expected that TTX would have no effect. TTX has also been shown to be unsuccessful in blocking the depolarizing responses of FMRamide in other Helix neurones such as D1, D2, D4, E14, E15 and F77 (Boyd and Walker, 1985).

4.5.2 LIGNOCAINE

The local anaesthetic lignocaine was applied to the C2 neurone. Such drugs block the transient increase in Na^+ permeability that generates the rising phase of the spike potential in response to depolarizing currents (Covine, 1971; Lechat, 1971). This Na^+ channel blocker had no effect on the amplitude of the FMRamide response.

4.5.3 AMILORIDE

Some Na^+ channels of the apical membranes of tight epithelia are blocked by low contrations of amiloride. Hamilton and Eaton (1985) found that amiloride blocks the channel from the outside and that this reduces the mean open time in a voltage-dependent manner without altering the single channel conductance. Amiloride has also been shown to block mechano-electrical transduction (MET) currents in dissociated hair cells

of the chick (Jorgensen and Ohmori, 1988). The transduction channels are non selective to monovalent cations, although it is thought that K^+ is the ion which carries the major fraction of the receptor current. The blocking by amiloride of this current was reversible and both dose and voltage dependent on the MET channel.

When $100\mu M$ amiloride was applied to the C2 neurone, the amplitude of the FMRFamide responses was almost completely blocked. This action of amiloride was fully reversible. Therefore, the most complete block in all of these experiments in terms of reducing the FMRFamide response amplitude and reversibility was achieved with amiloride.

4.6 EFFECT OF CYCLIC AMP ON THE FMRFamide RESPONSE OF THE C2 NEURONE

Song and Huang (1990) made an interesting discovery when examining glycine receptor channels in neurones isolated from the spinal trigeminal nucleus of the rat. These channels are thought to belong to a superfamily of ligand gated channels of which nicotinic ACh receptors and $GABA_A$ receptors belong. The Cl^- currents induced by glycine in these neurones were potentiated in the presence of elevated cyclic AMP levels and also by the activation of cyclic AMP dependent protein kinase A. The activation of a G_s protein was also involved in this potentiation. Since it is now known that the Na^+ channels opened by

FMRFamide in the C2 neurone are ligand gated (Cottrell et al, 1990), the question was, do elevated levels of cyclic AMP potentiate the FMRFamide response in the C2 neurone?

When 1mM dibutyryl cyclic AMP was applied to C2 neurones, FMRFamide responses were potentiated. The potentiation was reversed when the soluble analogue was washed away. A small inward current was induced by dibutyryl cyclic AMP. This was not surprising as discussed in relation to the C1 neurone when membrane soluble analogues of cyclic AMP induced an inward current. This is quite a common action of raised cyclic AMP levels in molluscan neurones. Overall these results suggest that the FMRFamide response is influenced in a positive manner by raised cyclic AMP levels.

Another membrane soluble analogue of cyclic AMP, 8-(-4) CPT cyclic AMP produced a larger potentiation of the FMRFamide response compared to dibutyryl cyclic AMP and a larger inward current. These large effects of 8-(-4) CPT cyclic AMP can be explained by the fact that this compound is more resistant to phosphodiesterase breakdown. Application of the phosphodiesterase inhibitor IBMX had similar effects to those of the membrane soluble analogues of cyclic AMP. Cyclic AMP itself, which is not membrane soluble, did not effect the FMRFamide response as was expected. These results suggest that raised cyclic AMP levels potentiate the FMRFamide response.

The question which remained after these experiments was, how does cyclic AMP potentiate the

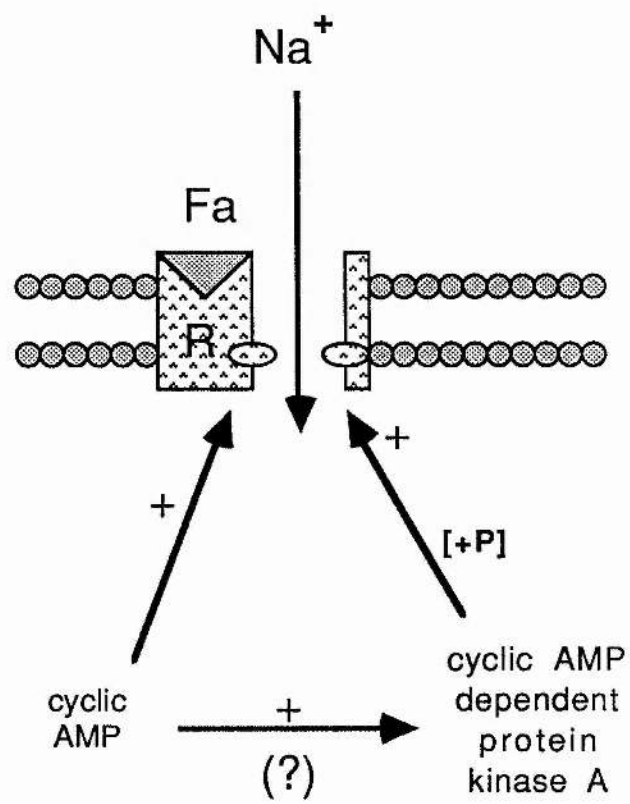
response? Do raised cyclic AMP levels activate cyclic AMP dependent protein kinase A to increase protein phosphorylation, or does cyclic AMP have some other effect on the receptor channel complex to potentiate the response? To investigate this, 8-(-4) CPT cyclic AMP was applied to C2 neurones simultaneously with okadaic acid and then the preparation was washed with okadaic acid alone. Okadaic acid inhibits protein phosphatases 1 and 2A (see above). The idea was that if raised cyclic AMP levels induced protein phosphorylation and potentiation of the FMRFamide response, okadaic acid would inhibit dephosphorylation and the potentiation of the FMRFamide response would be maintained.

When the combination of 8-(-4) CPT cyclic AMP and okadaic acid was applied to the neurones, the response was potentiated as usual. Washing the preparation with okadaic acid caused the response amplitudes to return to control levels. This result appeared to rule out protein phosphorylation as a mechanism to potentiate the FMRFamide response. A direct potentiation of the response seems more likely. A possible model of this is shown in Figure 4.3. However, it should be noted that a protein phosphatase other than the 1 or 2A type may be important and rapidly dephosphorylates the channels or their associated proteins. One way to overcome this problem would be to inject activated cyclic AMP dependent protein kinase A and see if the FMRFamide response was potentiated.

Figure 4.3

A schematic diagram of a possible mechanism underlying the potentiating effect of cyclic AMP on the FMRFamide induced fast depolarizing response of the C2 neurone. The evidence indicates that cyclic AMP has a direct effect on the protein(s) which make up the ligand gated channel. However, it is still possible that raised cyclic AMP levels may activate cyclic AMP dependent protein kinase A which could phosphorylate the channel proteins and cause the potentiation of the FMRFamide response.

The abbreviations used in this diagram are:-
R=receptor, Fa= FMRFamide, P= protein phosphorylation, += potentiation, activation of and increased levels of, as appropriate. The lipid bilayer of the cell membrane is represented by the shaded circles and the area between them.



The idea that cyclic AMP may directly potentiate the FMRFamide response is not unique. Recently Di Francesco and Tortora (1991) examined the activation of cardiac pacemaker channels by raised cyclic AMP levels. They found using excised patch experiments that cyclic AMP activated the hyperpolarization-activated current by a mechanism which did not involve protein phosphorylation. The effect of cyclic AMP involved a direct interaction with the channels on the cytoplasmic side. This action of cyclic AMP was found not to be mediated by a G protein.

4.7 OVERVIEW

Peptides are an important group of extracellular messengers. They occurred early in evolution and probably pre-dated some of the other classes of chemical messengers. Because of their structural diversity, peptide molecules offer an extensive vocabulary of signals. Up to 10-15 years ago it was thought that peptides were only used in different animal groups for long distance "chemically addressed" hormonal signalling. However, since then there has been an explosion of information showing that neuropeptides are present in the nervous system. Evidence from studies on both vertebrate and invertebrate animals indicates that many neuropeptides also have "transmitter-like" actions in that they can be "spatially addressed" close to their site of action. Therefore neurotransmission does not appear to be only

mediated by low molecular weight substances as was thought previously.

There are some problems in studying peptide functions especially within the CNS. The peptides generally occur in very low concentrations; immunocytochemical procedures which can be used to detect them are often insufficiently selective to identify a particular member of a peptide family and individual members of a peptide family often have markedly different effects to one another, sometimes even opposing actions. With vertebrate preparations it can be extremely difficult to examine the action of neuropeptides because the cells are small and heterogeneous. Therefore, experiments are best done on systems which are likely to yield unambiguous data such as simple preparations ideally comprising only a few identified cells with known properties that can be studied by a wide range of techniques.

The experiments detailed in this thesis have examined two different ionic currents induced by the neuropeptide FMRFamide in the mollusc Helix aspersa. The advantages of using this preparation are that this neuropeptide and others related to it are endogenous to the animal and that the neurones on which to study the actions of FMRFamide are large and readily identifiable.

The profile of the FMRFamide induced K^+ current in the C1 neurone is slow which suggests that the receptor is not directly linked to the ion channel. The evidence presented shows that a G_i or G_o protein is involved but

the response is not directly mediated through the action of a second messenger substance. It seems likely that the ion channel is linked to the receptor through the G protein. This effect of a neuropeptide on nervous system is similar to that described by the activation of ACh muscarinic receptors in atrial cardiac myocytes.

The FMRFamide response examined in the C2 neurone was unusual for a neuropeptide. Previous studies have shown that this fast Na^+ response is ligand-gated which is absolutely unique in the world of neuropeptides described to date. The receptor-channel complex may well be related to the superfamily of ligand gated channels, examples of which are the ACh nicotinic receptor and the GABA_A receptor. This relationship may be confirmed by molecular biological techniques and so prove to be an example of convergent evolution.

Characterization of the response in the C2 neurone showed that it was Ca^{2+} -independent and was blocked by amiloride. The response was shown to be potentiated by the second messenger cyclic AMP. This evidence indicates that ligand-gated channels may be modulated by intracellular second messenger levels, either by direct actions or possibly through protein phosphorylation. Overall the results detailed in this thesis illustrate how electrophysiological techniques may be used to examine the roles, mechanisms of action and receptors of neuropeptides in the nervous system.

Summary

1. FMRFamide induces a slow hyperpolarizing K^+ current in the C1 neurone and a fast depolarizing Na^+ current in the C2 neurone of Helix aspersa. Both effects were examined in this study.
2. The slow K^+ current of the C1 neurone was mimicked by the intracellular injection of the non-hydrolysable GTP analogue, GTP γ S. This substance also occluded the FMRFamide response. A G protein therefore appears to be involved in the response.
3. Pre-activated pertussis toxin injected into the cell body of the C1 neurone reduced the amplitude of the FMRFamide response which indicated that the G protein involved was of the G_i or G_o type.
4. Raised cyclic AMP levels produced by application of the membrane soluble analogue 1mM 8-(-4) CPT cyclic AMP caused a reduction in the amplitude of the FMRFamide response of the C1 neurone and the development of an inward current.
5. 100 μ M 5-HT also produced a reduction in FMRFamide response amplitude of the C1 neurone and an inward current. Both of these effects were potentiated and prolonged by the simultaneous application of the phosphodiesterase inhibitor 1mM IBMX, which indicated that 5-HT acted through cyclic AMP production. The channels opened by FMRFamide and closed by 5-HT may be an "S" K^+ type channel.

6. A membrane soluble analogue of cyclic GMP, 1mM 8-bromo cyclic GMP had no effect on the amplitude of the FMRFamide response of the C1 neurone, but did produce an inward current.

7. Injection of IP_3 into the neuronal cell body had no effect on the baseline current level of the C1 neurone.

8. Activation of protein kinase C by 50 μ M phorbol 12,13-dibutyrate irreversibly reduced the amplitude of the FMRFamide response of the C1 neurone and produced a reversible inward current.

9. Application of 50 μ M arachidonic acid to the C1 neurone had no effect on the amplitude of the FMRFamide response and did not mimic the response.

10. Experiments designed to reduce intracellular Ca^{2+} concentration either by injecting the Ca^{2+} chelator EGTA, or removing Ca^{2+} from the extracellular environment, had no effect on the amplitude of the FMRFamide response of the C1 neurone. These treatments did induce an inward current, which may have been due to the closure of Ca^{2+} dependent K^+ channels.

11. Application of 1 μ M okadaic acid, the protein phosphatase 1 and 2A inhibitor, reduced the amplitude of the FMRFamide response of the C1 neurone indicating that increased protein phosphorylation levels make it more difficult for FMRFamide to open the K^+ channels.

12. Patch clamp experiments with cell attached patches on the C1 neurone indicated that at patch potentials

near resting membrane potential (-45mV), unitary currents were not observed. Unitary currents did become obvious at more depolarized patch potentials. They were thought to be voltage dependent K^+ channels. FMRFamide application at patch potentials near resting membrane potential did not result in the occurrence of outward unitary currents.

13. The fast depolarizing response of FMRFamide in the C2 neurone was characterized. The current was shown to be mainly carried by Na^+ since the response was almost abolished with ONa^+ saline. Blockage of the Ca^{2+} channels by 1mM CoCl_2 did not affect the response.

14. $100\mu\text{M}$ amiloride reversibly blocked the FMRFamide response of the C2 neurone.

15. Application of the Na^+ channel blocker tetrodotoxin ($50\mu\text{M}$) and the local anaesthetic lignocaine (1mM) had no effect on the FMRFamide response of the C2 neurone.

16. The membrane soluble analogues of cyclic AMP, 1mM dibutyryl cyclic AMP and 1mM 8-(-4) CPT cyclic AMP reversibly potentiated the FMRFamide response of the C2 neurone. 1mM IBMX had the same action on the response. All of these treatments also induced inward currents which were not examined in detail.

17. When the C2 neurone was bathed in 1mM 8-(-4) CPT cyclic AMP simultaneously with $1\mu\text{M}$ okadaic acid and then washed with $1\mu\text{M}$ okadaic acid alone, the FMRFamide response was reversibly potentiated. The increased

protein phosphorylation levels induced by okadaic acid had no effect. Thus the effect of raised cyclic AMP levels may be direct and not involve protein phosphorylation.

References

ADAMS, P.R., BROWN, D.A., & JONES, S.W. (1983). Substance P inhibits the M-current in bullfrog sympathetic neurones. *British J. Pharmacology* 79 : 330-333.

AKAIKE, N., BROWN, A.M., NISHI, K., & TSUDA, Y. (1981). Actions of verapamil, diltiazem and other divalent cations on the calcium current of Helix neurones. *British J. Pharmacology* 74 : 87-95.

ARAI, H., & EMSON, P.C. (1986). Regional distribution of neuropeptide K and other tachykinins (neurokinin A, neurokinin B and substance P) in rat central nervous system. *Brain Research* 399 : 240-249.

ALVAREZ-LEEFMANS, F.J., & GAMINO, S.M. (1982). Intracellular free potassium concentration in snail neurones measured with ion-sensitive micro-electrodes. *Soc. Neuroscience Abst.* 8:684.

BAILE, C., MCLAUGHLIN, C., & DELLA-FERA, M.A. (1986). Role of cholecystokinin and opioid peptides in the control of food intake. *Physiol Rev.* 66 : 172-202.

BEINFELD, M.C., MEYER, D.K., ESKAY, R.L., JENSEN, R.T., & BRAINSYEN, M.J. (1981). The distribution of CCK immunoreactivity in the central nervous system of the rat as determined by RIA. *Brain Research* 212 : 51-57.

BELARDETTI, F., KANDEL, E.R., & SIEGELBAUM, S.A. (1987). Neuronal inhibition by the peptide FMRFamide involves opening of $S K^+$ channels. *Nature* 325 : 153-156.

BENOS, D. (1982). Amiloride: a molecular probe of sodium transport in tissues and cells. *American J. Physiol.* 242 : C131-C145.

BEWICK, G.S., PRICE, D.A., & COTTRELL, G.A. (1990). The fast response mediated by the C3 motoneurone of Helix is not attributable to the contained FMRFamide. J. Exp. Biol. 148 : 201-219.

BIALOJAN, C., & TAKAI, A. (1988). Inhibitory effect of a marine sponge toxin, okadaic acid, on protein phosphatases specificity and kinetics. J. Biochemistry 256 : 283-290.

BIRNBAUMER, L., ABRAMOWITZ, J., & BROWN, A. (1990). Receptor-effector coupling by G-proteins. Biochimica et Biophysica Acta 1031 : 163-224.

BODEN, P., & HILL, R.G. (1988). Effects of cholecystokinin and related peptides on neuronal activity in the ventromedial nucleus and hypothalamus. British J. Pharmacology 94 : 246-252

BOURNE, H.R. (1986). One molecular machine can transduce diverse signals. Nature 321 : 814-816.

BOYD, P.J., & WALKER, R.J. (1985). Actions of the molluscan neuropeptide FMRFamide on neurons in the suboesophageal ganglia of the snail Helix aspersa. Comp. Biochem. Physiol. 81C(2) : 379-386.

BOYD, P.J., & WALKER, R.J. (1987). Comparison of the effects of FMRFamide and pQDPFLRFamide on identified Helix neurons. Comp. Biochem. Physiol. 86C(2) : 371-373.

BRANTON, W.D., PHILLIPS, H.S., & JAN, Y.N. (1986). The LHRH family of peptide messengers in the frog nervous system. Progress in Brain Research 205-215. Eds T.Hokfelt, K.Fuxe and B.Pernav. Elsevier. Amsterdam 1986.

BREZINA, V. (1988). Guanosine 5'- triphosphate analogue activates potassium current modulated by neurotransmitters in Aplysia neurones. J. Physiol. 407 : 15-40.

BREZINA, V., ECKERT, R., & ERXLEBEN, C. (1987a). Modulation of potassium conductances by an endogenous neuropeptide in neurons of Aplysia californica J. Physiol. 382 : 267-290.

BREZINA, V., ECKERT, R., & ERXLEBEN, C. (1987b). Suppression of calcium current by endogenous neuropeptide in neurons of Aplysia californica. J. Physiol. 388 : 565-595.

BROWN, R.V., BASBAUM, A.I., & MAYERI, E. (1984). Identification of FMRF-amide immunoreactive neurones in the abdominal ganglion of Aplysia. Soc. Neuroscience Abst. 10 : 691

BULLOCH, G.M., PRICE, D.A., MURPHY, A.D., LEE, T.D., & BOWES, H.N. (1988). FMRFamide peptides in Helisoma: Identification and physiological actions at a peripheral synapse. J. Neuroscience 8 : 3459-3469.

BURGESS, G.M., MULLANEY, I., McNEIL, M., DUNN, P.M., & RANG, H.P. (1989). Second messengers involved in the mechanism of action of bradykinin in sensory neurons in culture. J. Neuroscience 9(9) : 3314-3325.

BUTTNER, N., SIEGELBAUM, S.A., & VOLTERRA, A. (1989). Direct modulation of Aplysia S- K⁺ channels by a 12- lipxygenase metabolite of arachidonic acid. Nature 342 : 553-555.

CALLOWAY, J.C., MASINOVSKY, B., & GRAUBARD, K. (1987). Co-localization of SCP-like and FMRFamide-like immunoreactivities in crustacean nervous systems. Brain Research 405 : 295-304.

CARDOT, C & FELLMAN, D. (1983). Immunofluorescent evidence of an FMRFamide-like peptide in the peripheral nervous system of the gastropod mollusc Helix aspersa. Neuroscience Letters 43 : 167-172.

CARLBERG, M., MONSW, N., GREFFARD, M. & NASSEL, D.R. (1989). L-DOPA and FMRFamide immunoreactivity in the tentacular nerve plexus of the sea anemone Metridium senile. Comp. Biochem. Physiol. 94C(2) : 435-440.

CARPENTER, D.O., SWANN, J.W., & YAROWSKY, P.J. (1977). Effect of curare on responses to different putative neurotransmitters in Aplysia neurones. J. Neurobiology 8(2) : 119-132.

CARROLL, L.S., CARROW, G.M., & CALABRESE, R.L. (1986). Localization and release of FMRFamide-like immunoreactivity in the cerebral neuroendocrine system of Manduca sexta. J. Exp. Biol. 126 : 1-14.

CHANG, M.M., LEEMAN, S.E., & NIAL, H.D. (1971). Amino acid sequence of substance P. Nature, New Biol. 232 : 86-87.

CHIBA, A., OKA, S., & HONMAN, Y. (1991). Immunocytochemical distribution of FMRFamide-like substance in the brain of the cloudy dogfish, Scyliorhinus torazame. Cell Tissue Res. (1991) 265 : 243-250.

COHEN, P., COTTRELL, G.A., & HILL-VENNING, C. (1989). The protein phosphatase inhibitor okadaic acid potentiates the 5-HT induced suppression of a K current in the C1 neurone of Helix aspersa. J. Physiol. 415 : 33P

COLOMBAIONI, L., PAUPARDIN-TRITSCH, D., VIDAL, P.P., & GERSCHENFELD, H.M. (1985). The neuropeptide FMRFamide decreases both the Ca^{2+} conductance and a

cyclic 3',5'-Adenosine monophosphate-dependent K^+ conductance in identified molluscan neurons. J. Neuroscience 5(9) : 2533-2538.

CONNOR, J.A., & HOCKBERGER, P. (1984). A novel membrane sodium current induced by injection of cyclic nucleotides into Gastropod neurons. J. Physiol. 354 : 139-162.

COREY, D.P., & STEVENS, C.F. (1983). Science and technology of patch-recording electrodes. In Single-channel recording. Ed. B.Sskmann, and E.Neher, Plenum Press.

COTTRELL, G.A. (1982). FMRFamide neuropeptides simultaneously increase and decrease K^+ currents in an identified neuron. Nature 296(5852) : 87-89.

COTTRELL, G.A., & DAVIES, N.W. (1987). Multiple receptor sites for a molluscan peptide (FMRFamide) and related peptides of Helix. J. Physiol. 382 : 51-68.

COTTRELL, G.A., DAVIES, N.W., & GREEN, K.A. (1984). Multiple actions of a molluscan cardioexcitatory neuropeptide and related peptides on identified Helix neurons. J. Physiol. 356 : 315-333.

COTTRELL, G.A., & GREEN, K.A. (1984). Single-channel analysis of the action of FMRFamide on K channels in Helix neurons. J. Physiol. 346 : 97P.

COTTRELL, G.A., GREEN, K.A., & DAVIES, N.W. (1990). The neuropeptide Phe-Met-Arg-Phe-NH₂ (FMRFamide) can activate a ligand-gated ion channel in Helix neurones. Pflugers Arch. 416 : 612-614.

COTTRELL, G.A., & LESSER, W. (1987). A comparison of the effect of some molluscan (FMRFamide-like) peptides on the calcium current of a snail neurone. J. Physiol. 392 : 33P.

COTTRELL, G.A., PRICE, D.A., & GREENBERG, M.J. (1981). FMRFamide-like activity in the ganglia and in a single identified neurone of Helix aspersa. Comp. Biochem. Physiol. 70C : 103-107.

COTTRELL, G.A., SCHOT, P.C., & DOCKRAY, G.J. (1983). Identification and probable role of a single neuron containing the neuropeptide Helix FMRFamide. Nature 304 (5927) : 638-640.

COVINO, B.G. (1971). Comparative clinical pharmacology of local anaesthetic agents. Anesthesiology 35 : 158-167.

CRITZ, S.D., BAXTER, D.A., & BYRNE, J.H. (1991). Modulatory effects of serotonin, FMRFamide, and myomodulin on the duration of action potentials, excitability, and membrane currents in tail sensory neurones of Aplysia. J. Neurophysiol. 66(6) : 1912-1926.

DAVENPORT, T.R.B., LEE, D.L., & ISAAC, R.E. (1988). Immunocytochemical demonstration of a neuropeptide in Ascaris suum (Nematoda) using an antiserum to FMRFamide. Parasitology 97 : 81-88.

DAVIES, N.W. (1986). An electrophysiological investigation of the actions of FMRFamide and related peptides on neurones of Helix aspersa. Ph.D Thesis. University of St Andrews.

DAY, N.C., HALL, M.D., CLARK, C.R., & HUGHES, T. (1986). High concentrations of cholecystokinin binding sites in the ventromedial nucleus of the hypothalamus. Neuropeptides 8 : 1-18.

DiFRANCESCO, D., & TORTORA, P. (1991). Direct activation of cardiac pacemaker channels by intracellular cyclic AMP. Nature 351 : 145-147.

DOCKRAY, G.J., REEVE, J.R.Jr., SHIVELY, J., GAYTON, R.J., & BARNARD, C.S. (1983). A novel active pentapeptide from chicken brain identified by antibodies to FMRFamide. *Nature* 305 : 328-330.

DODD, P.R., EDWARDSON, J.A., & DOCKRAY, G.J. (1980). The depolarization-induced release of CCK C-terminal octapeptide (CCK-8S) from rat synaptosomes and brain slices. *Regul. Pept.* 1 : 17-29.

EBBERINK, R.H.M., PRICE, D.A., VAN LOOENHONT, H., DOBLE, K.E., REIHM, J.P., GERAERTS, W.P.M., & GREENBERG, M.H. (1987). The brain of Lymnea contains a family of FMRFamide-like peptides. *Peptides* 8 : 515-522.

ELEKES, K., & NASSEL, D.R. (1990). Distribution of FMRFamide-like immunoreactive neurons in the central nervous system of the snail Helix pomatia. *Cell Tissue Res.* 262 : 177-190.

EL-FAKAHANY, E.E., ALGER, B.E., LAI, W.S., PITLER, I.A., WORLEY, P.F., & BARABAN, J.M. (1988). Neuronal muscarinic responses: role of protein kinase C. *FASEB J.* 2 : 2575-2583.

ERSPAMER, V., & ANASTASI, A. (1962). Structure and pharmacological actions of eledoisin, the active endecapeptide of the posterior salivary glands of Eledone. *Experientia* 18 : 58-59.

EWALD, D.A., MATTIES, J.G., PERNEY, T.M., WALKER, M.W., & MILLER, R.J. (1988). The effect of down regulation of protein kinase C on the inhibitory modulation of dorsal root ganglion neurone Ca^{2+} currents by neuropeptide Y. *J. Neuroscience* 2447-2451.

FINK, L., CONNOR, J.A., STRONG, J.A., & KACZMAREK, L.K. (1985). Inositol trisphosphate

injections and imaging of the calcium indicator fura-2 in molluscan neurones. Soc. Neuroscience Abst. 11 : 854.

FRONTALI, N., WILLIAMS, L., & WELSH, J.H. (1967). Heart excitatory and inhibitory substances in molluscan ganglia. Comp. Biochem. Physiol. 22 : 833-841.

FUJIWARA- SAKATA, M., MUNEOKA, Y., & KOBAYASHI, M. (1991). Action and immmmunoreactivity of neuropeptides in the buccal neuromuscular system of a prosobranch mollusc, Rapana thomasiana. Cell Tissue Res. 264 : 57-62.

GILLETTE, R., & GREEN, D.J. (1987). Calcium dependence of votage-sensitivity in adenosine 3',5'-cyclic monophosphate stimulated sodium current in Pleurobranchaea. J. Physiol. 393 : 233-245.

GINTZLER, A.R., & XU, H. (1991). Different G proteins mediate the opioid inhibition or enhancement of evoked [5-methionine] enkephalin release. Proc. Natl. Acad. Sci. U.S.A. 88 : 4741-4745.

GREENBERG, M.J., PAYZA, K., NACHMAN, R.J., HOLMAN, G.M., & PRICE, D.A. (1988). Relationships between the FMRFamide-related peptides and other peptide families. Peptides 9(1) : 125-135

GRIMMELIKHUIJZEN, C.J.P., HAHN, M., RINEHART, K.L., & SPENCER, A.N. (1988). Isolation of <Glu-Leu-Leu-Gly-Gly-Arg-Phe-NH₂ (Pol-RFamide)>, a novel neuropeptide from hydromedusae. Brain Research 475 : 198-203.

GRIMMELIKHUIJZEN, C.J.P., & SPENCER, A.N. (1984). FMRFamide immunoreactivity in the nervous system of the Medusa Polyorchis penicillatus. J. Comp. Neurology 230 : 361-371.

GRIMMELIKHUIJZEN, C.J.P., & GRAFF, D. (1986). Isolation of <Glu-Gly-Arg-Phe-NH₂ (Antho-RFamide)>, a neuropeptide from sea anemones. Proc. Natl. Acad. Sci. USA 83 : 9817-9821.

HALPAIN, S., GIRAULT, J-A., & GREENGARD, P. (1990). Activation of NMDA receptors induces dephosphorylation of DARP-32 in rat striatal slices. Nature 343 : 369-372.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B., & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell free membrane patches. Pflugers Arch. 391 : 85-100.

HAMILTON, K.L., & EATON, D. (1985). Single channel recordings from an amiloride-sensitive epithelial sodium channel. American J. Physiol. 249 : C200-C207.

HAMMOND, C., PAUPARDIN-TRITSCH, D., NAIRN, A.C., GREENGARD, P., & GERSCHENFELD, H.M. (1987). Cholecystokinin induces a decrease in Ca²⁺ current in snail neurons that appears to be mediated by protein kinase C. Nature 325 : 809-811.

HANLEY, M.R., LEE, C.M., JONES, L.M., & MICHELL, R.H. (1980). Similar effects of substance P and related peptides on salivation and on phosphatidylinositol turnover in rat salivary glands. Molec.Pharmac. 18 : 78-83.

HAYDON, P.G., MAN-SON-HING, H., DOYLE, R.T., & ZORAN, M. (1991). FMRFamide modulation of secretory machinery underlying presynaptic inhibition of synaptic transmission requires a pertussis-toxin-sensitive G-protein. J. Neuroscience 11 (12) : 3851-3860.

HENDERSON, G., HUGHES, J., & KOSTERLITZ, H.W. (1978). In vitro release of Leu- and Met- enkephalin from the corpus striatum. *Nature* 271 : 677-679.

HIGASHIDA, H., & BRAUN, D.A. (1986). Two polyphosphatidylinositide metabolites control two K^+ currents in a neuronal cell. *Nature* 323 : 333-335.

HILL-VENNING, C. & COTTRELL, G.A. (1988). C-kinase and the slow voltage dependent response to serotonin in a serotonergic neurone (GSN) of the snail. *Soc. Neuroscience Abst.* 14,130.

HOCKBERGER, P., & CONNOR, J.A. (1984). Alteration of calcium conductances and outward current by cyclic adenosine monophosphate (cAMP) in neurones of Limax maximus. *Cell. Molec. Neurobiol.* 4 : 319-338.

HODGKIN, A.L., & KATZ, B. (1949). The effect of sodium ions on the electrical activity of the giant axon of the squid. *J. Physiol.* 108 : 37-77.

HOLMAN, M., COOK, B.J., & NACHMAN, R.J. (1986). Isolation, primary structure and synthesis of leucomyosuppressin, an insect neuropeptide that inhibits spontaneous contractions of cockroach hindgut. *Comp. Biochem. Physiol.* 85C : 329-333.

HOKFELT, T., KELLERTH, O.D., NILLSON, G., & PERNOW, B. (1975). Experimental immunohistochemical studies on the localization and distribution of substance P in rat primary sensory neurones. *Brain Research* 100 : 235-252.

HSIA, J.A., MOSS, T., HEWLETT, E.L., & VAIGHAN, M. (1984). ADP-ribosylation of adenylate cyclase by pertussis toxin. Effects on inhibitory agonist binding. *J. Biol. Chem.* 259 : 1086-1090.

HUGHES, J., SMITH, T.W., KOSTERLITZ, H.W.,
FOTHERGILL, L.A., MORGAN, B.A., & MORRIS, H.R. (1975).
Identification of two related pentapeptides from the
brain with potent opiate agonist activity. *Nature* 258
: 577-579.

ICHINOSE, M., & BYRNE, J.H. (1991). Role of
protein phosphatases in the modulation of neuronal
membrane currents. *Brain Research* 549 : 146-150.

ICHINOSE, M., & McADOO, D.J. (1988). The
voltage- dependent, slow inward current induced by the
neuropeptide FMRFamide in Aplysia neuron R14.
J. Neuroscience 8(10) : 3891-3900.

ICHINOSE, M., & McADOO, D. (1989). The cyclic
GMP- induced inward current in neuron R14 of Aplysia
californica : similarity to a FMRFamide-induced inward
current. *J. Neurobiology* 20(1) : 10-24.

IVERSEN, L.L. (1983). Nonopioid neuropeptides in
mammalian CNS. *Ann.Rev.Pharmacol.Toxicol* 23 : 1-27.

JORGENSEN, F., & OHMORI, H. (1988). Amiloride
blocks the mechano-electrical transduction channel of
hair cells of the chick. *J. Physiol.* 403 : 577-588.

KANAZAWA, I., OGAWA, T., KIMURA, S., & MUNEKATA,
E. (1984). Regional distribution of substance P,
neurokinin α and neurokinin B in rat central nervous
system. *Neuroscience. Res.* 2 : 111-120.

KATADA, T., GILMAN, A.G., WATANABE, Y., BAUER, S.,
& JAKOBS, I. (1985). Protein kinase C phosphorylates
the inhibitory guanine-nucleotide-binding regulatort
component and apparently suppresses its function in
hormonal inhibition of adenylate cyclase. *Eur. J.*
Biochem. 151 : 431-437.

- KAZMI, S.M.I., & MISHRA, R.K. (1987). Comparative pharmacological properties and functional coupling of μ and δ opioid receptor sites in human neuroblastoma SH-SY5Y cells. *Molecular Pharmacology* 32 : 109-188.
- KIMURA, S., OKADA, M., SUGITAL, Y., KANAZAWA, I., & MUNEKATA, E. (1983). Novel neuropeptides, neurokinin α and β isolated from porcine spinal cord. *Proc. Japan. Acad.* 59B : 101-104.
- KIVIPELTO, L., & PANULA, P. (1990). Comparative distribution of neurons containing FLFQPQRFamide-like (morphine-modulating) peptide and related neuropeptides in the rat brain. *Euro. J. Neuroscience* 3 : 175-185.
- KOIZANI, O., WILSON, J.D., GRIMMELIKHUIJZEN, C.J.P., & WESTFALL, J.A. (1989). Ultrastructural localization of RF-amide-like peptides in neuronal dense-core vesicles in the Penduncle of Hydra. *J. Exp. Zoology* 249 : 17-22.
- KONISHI, S., & OTSUKA, M. (1974). Excitatory action of hypothalamic substance P on spinal motoneurons of new born rats. *Nature* 252 : 734-735.
- KRAVITZ, E.A., KOBIERSKI, L., TRIMMER, B.A., & GOY, M.F. (1987). Peptide F₁ : a myoactive lobster peptide related to FMRF-amide. *Soc. Neuroscience Abst.* 13 : 1257.
- KURACHI, Y., NAKAJIAMA, T., & SUGIMOTO, T. (1986a). Acetylcholine activation of K⁺ channels in cell-free membrane of atrial cells. *American J. Physiol.* 251 H681-684.
- KURACHI, Y., NAKAJIMA, T., & SUGIMOTO, T. (1986b). On the mechanism of activation of muscarinic K⁺ channels by adenosine in isolated atrial cells:

involvement of GTP-binding proteins. Pflugers Arch.
407 : 264-274.

LARSSON, L-I., & REHFELD, J. (1979).
Localization and molecular heterogeneity of
cholecystokinin in the central and peripheral nervous
system. Brain Research 347 : 1-10.

LECHAT, P. (1971). Local anaesthetics.
International Encyclopedia of Pharmacology and
Therapeutics. Section 8, Vol 1. Pergamon Oxford.

LEHMAN, H.K., & PRICE, D.A. (1987). Localization
of FMRFamide-like peptides in the snail Helix aspersa.
J. Exp. Biol. 131 : 37-53.

LEHMAN, H.K., PRICE, D.A., & GREENBERG, M.J.
(1984). The FMRFamide-like neuropeptide of Aplysia is
FMRFamide. Biol. Bull. 167 : 460-466.

LESSER, W. (1989). Actions and distributions of
cardioactive peptides in two molluscs. Ph.D. Thesis.
University of St Andrews.

LINACRE, A., KELLETT, E., SAUNDERS, S., BRIGHT,
K., BENJAMIN, P.R., & BURKE, J.F. (1990).
Cardioactive neuropeptide Phe-Met-Arg-Phe-NH₂
(FMRFamide) and novel related peptides are encoded in
multiple copies by a single gene in the snail Lymnaea
stagnalis. J. Neuroscience 10(2) : 412-419.

LINDERFORS, N., BRODIN, E., THEODORSSON-NORHEIM,
E., & UNGERSTEDT, U. (1985). Calcium dependent
potassium-stimulated release of neurokinin A and
neurokinin B from rat brain in vitro. Neuropeptides 6 :
453-461.

LORD, J.A.H., WATERFIELD, A.A., HUGHES, J., &
KOSTERLITZ, H.W. (1977). Endogenous opioid peptides:
multiple agonists and receptors. Nature 267 : 495-499.

LUTZ, E., McDONALD, M., HETTLE, S., COTTRELL, G.A., & SOMMERVILLE, J. (1991). Structure and expression of messenger RNA molecules encoding FMRFamide-related peptides in Helix aspersa. In Press.

MCCROHAN, C.R., & GILLETTE, R. (1988). Cyclic AMP-stimulated sodium current in identified feeding neurones of Lymnea stagnalis. Brain Research 438 : 115-123.

MANCILLAS, J.R., SIGGINS, G.R., & BLOOM, F.E. (1986). Somatostatin selectively enhances acetylcholine-induced excitations in rat hippocampus and cortex. Proc. Natl. Acad. Sci. U.S.A. 83 : 7518-7521.

MAN-SON-HING, H., ZORAN, M.J., LUKOWIAK, K., & HAYDON, P.G. (1989). A neuromodulator of synaptic transmission acts on the secretory apparatus as well as on ion channels. Nature 341 : 237-239.

MARTIN, W.R., EADES, C.G., THOMSON, J.A., HAPPHER, R.E., & GILBERT, P.E. (1976). The effects of morphine and nalorphine-like drugs in the non-dependent and morphine-dependent chronic dependent drugs. J. Pharmac. Exp. Ther 197 : 517-532.

MASU, Y., NAKAYAMA, K., TAMAKI, H., HARADA, Y., KUNO, M., & NAKAMISHI, S. (1987). cDNA cloning of bovine-K receptor through oocyte expression system. Nature 329 : 836-838.

MATSUTO, T., YANAGISAWA, M., OTSUKA, M., KANAZAWA, I., & MUNEKATA, t. (1984). The excitatory action of newly discovered mammalian tachykinins, neurokinin α and neurokinin β , on neurons of the isolated spinal cord of the new-born rat. Neuroscience. Res. 2 : 105-110.

MEECH, R.W. (1978). Calcium dependent potassium activation in nervous tissues. Ann. Rev. Biophys. Bioeng. 7 : 1-18.

MERCIER, A.J., ORCHARD, I., & TEBRUGGE, V. (1991). FMRFamide-like immunoreactivity in the crayfish nervous system. J. Exp. Bio. 156 : 519-538.

MILLER, J.P., BECK, A.H., SIMON, L.N., & MEYER, R.B. (1975). Induction of hepatic tyrosine aminotransferase in vivo by derivatives of cyclic 3',5' monophosphate. J. Biol. Chem. 250 : 426-431.

MOORE, J.W., BLAUSTEIN, M.P., ANDERSON, N., & NARAHASHI, T. (1967). Basis of tetrodotoxins selectivity in blockage of squid axons. J. Gen. Physiol. 50 : 401-411.

MUSKE, L.E., DOCKRAY, G.J., CHOHAN, K.S., & STELL, W.K. (1987). Segregation of FMRFamide-immunoreactive efferent fibres from NPY-immunoreactive amacrine cells in Golfish retina. Cell Tissue Res. 247 : 299-307.

NAGLE, G.T. (1982). The molluscan neuropeptide FMRFamide : calcium-dependent release levels in Macrocallista (Bivalvia). Life Science 30 : 803-807.

NAMBU, J.R., MURPHY-ERDOSH, C., ANDREWS, P.C., FEISTNER, G.J., & SCHELLER, R.H. (1988). Isolation and characterization of a drosophila neuropeptide gene. Neuron 1 : 55-61.

NEER, E.J., LOCK, J.M., & WOLF, L.G. (1984). Purification and properties of the inhibitory guanine nucleotide regulatory unit of brain adenylate cyclase. J. Biol. Chem. 259 : 14222-14229.

NEER, E.J., & CLAPHAM, D.E. (1988). Roles of G protein subunits in transmembrane signalling. *Nature* 333 : 129-134.

NORTH, R.A., WILLIAMS, J.T., SAPRENANT, A., & CHRISTIE, M.J. (1987). μ and δ receptors belong to a family of receptors that are coupled to potassium channels. *Proc. Natl. Acad. Sci. U.S.A.* 84 : 5487-5491.

ORGAD, S., BREWIS, N.D., ALPHEY, L., AXTON, J.M., DUDAI, Y., & COHEN, P.T.W. (1990). The structure of protein phosphatase 2A is as highly conserved as that of protein phosphatase 1. *FEBS* 275(1,2) : 44-48.

ORON, Y., DASCAL, N., NADLER, E., & LUPU, M. (1985). Inositol 1,4,5-trisphosphate mimics muscarinic response in Xenopus oocyte. *Nature* 311 : 141-143.

OSTHHOLM, T., EKSTRAM, P., & EBBESSON, S.O.E. (1990). Distribution of FMRFamide-like immunoreactivity in the brain, retina and nervus terminalis of the sockeye salmon parr, Oncorhynchus nerka. *Cell Tissue Res.* 261 : 403-418.

OTSUKA, M., KONISHI, S., & TAKAHASHI, T. (1972). The presence of motoneurone-depolarizing peptide in bovine dorsal roots of spinal cord. *Proc. Japan. Acad.* 48 : 342-346.

PAUPARDIN-TRITSCH, D., HAMMOND, C., & GERSCHENFELD, H.M. (1986a). Serotonin and cyclic GMP induce an increase of the calcium current in the same identified molluscan neurones. *J. Neuroscience* 6(9) : 2715-2723.

PAUPARDIN-TRITSCH, D., HAMMOND, C., GERSCHENFELD, H.M., NAIRN, A.C., & GREENGARD, P. (1986b). cGMP-dependent protein kinase enhances Ca^{2+} current and

potentiates the serotonin induced Ca^{2+} current increase in snail neurones. *Nature* 323 :812-813.

PAUPARDIN-TRITSCH, D., & GERSCHENFELD, H.M. (1990). Serotonin induces a cyclic AMP-mediated outwardly rectifying slow K^+ current in a single identified snail neuron. *Euro. J. Neuroscience* 2 : 813-821.

PELLMAR, T.C. (1981). Ionic mechanism of a voltage dependent current elicited by cyclic AMP. *Cell. Molec. Neurobiol.* 1 : 87-97.

PFAFFINGER, P.J., MARTIN, J.M., HUNTER, D.D., NATHANSON, N.M., & HILLE, B. (1985). GTP-binding proteins couple cardiac muscarinic receptors to a K channel. *Nature* 317 : 536-537.

PIOMELLI, D., VOLTERRA, A., DALE, N., SIEGELBAUM, S.A., SCHWARTZ, J.H., & BELARDETTI, F. (1987). Lipoxigenase metabolites of arachidonic acid as second messengers for presynaptic inhibition of Aplysia sensory cells. *Nature* 328(6125) : 38-43.

PIOMELLI, D., SHAPIRO, E., ZIPKIN, R., SCHWARTZ, J.H., & FIENMARK, S.J. (1989). Formation and action of 8-hydroxy-11,12-epoxy-5,9,14-icosatrienoic acid in Aplysia : a possible second messenger in neurones. *Proc. Natl. Acad. Sci. U.S.A.* 86 : 1721-1725.

PONDAVEN, P., & COHEN, P. (1987). Identification of protein phosphatases-1 and 2A and inhibitor-2 in oocytes of the starfish Asterias rubens and Marthasterias glacialis. *Euro. J. Biochem.* 167 : 135-140.

PRENTKI, M., BIDEN, T.J., JANJIE, D., IRVINE, R.F., BERRIDGE, M.J., WOLLHEUM, C.B. (1984). Rapid mobilization of Ca^{2+} from rat insulinoma microsomes by inositol 1,4,5-trisphosphate. *Nature* 309 : 562-564.

PRICE, D.A., COTTRELL, G.A., DOBLE, K.E., GREENBERG, M.J., JORENBY, W., LEHMAN, H.K., & RIEHM, J.P. (1985). A novel FMRFamide-related peptide in Helix : pQDPFLRFamide. Biol. Bull. 169 : 256-266.

PRICE, D.A., & GREENBERG, M.J. (1977). The structure of a molluscan cardioexcitatory neuropeptide. Science 197 : 670-671.

PRICE, D.A., LESSER, W., LEE, T.D., DOBLE, K.E., & GREENBERG, M.J. (1990). Seven FMRFamide-related and two SCP-related cardioactive peptides from Helix. J. Exp. Biol. 154 : 421-437.

REUTER, M., KARHI, T., & SCHOT, L.P.C. (1984). Immunocytochemical demonstration of peptidergic neurones in the central and peripheral nervous systems of the flatworm Microstomum lineare with antiserum to FMRFamide. Cell Tissue Res. 238 : 431-436.

RICHMOND, J.E., FINTE, L.R., SMITH, W.L., PRICE, D.A., & HAYDON, P.G. (1991). Activation of a peptidergic synapse locally modulates postsynaptic calcium influx. J. Exp. Biol. 161 : 257-271.

ROBB, S., PACKMAN, L.C., & EVANS, P.D. (1989). Isolation, primary structure and bioactivity of SCHISTOFLRF-amide, a FMRFamide-like neuropeptide from the locust, Schistocerca gregaria. Biochem. Biophys. Res. Commun. 160(2) : 850-856.

ROBB, S., & EVANS, P.D. (1990). FMRFamide-like peptides in the locust : distribution, partial characterization and bioactivity. J. Exp. Biol. 149 : 335-360.

RODBELL, M. (1980). The role of hormone receptors and GTP-regulatory proteins in membrane transduction. Nature 284 : 17-22.

SAUNDERS, S.E., BRIGHT, K., KELLETT, E., BENJAMIN, P.R., & BURKE, J.F. (1991). Neuropeptides Gly-Asp-Pro-Phe-Leu-Arg-Phe-amide (GDPFLRFamide) and Ser-Asp-Pro-Phe-Leu-Arg-Phe-amide (SDPFLRFamide) are encoded by an exon 3' to Phe-Met-Arg-Phe-NH₂ (FMRFamide) in the Lymnaea stagnalis. J. Neuroscience 11(3) : 740-745.

SASAKI, K., & SATO, M. (1987). A single GTP-binding protein regulates K⁺ channels coupled with dopamine, histamine, and acetylcholine receptors. Nature 325 : 259-262.

SASAKI, K., TAKAHASHI, J., MATSUMOTO, M., TAKASHIMA, K., HAKOZAKI, S., & SATO, M. (1987). Islet activating protein-sensitive guanosine triphosphate-binding protein regulates K⁺ channels coupled with FMRFamide receptors. Japanese J. Physiol. 37 : 551-557.

SASEK, C.A., & ELDE, R.P. (1985). Distribution of neuropeptide Y-like immunoreactivity and its relationship to FMRFamide-like immunoreactivity in the sixth lumbar and first sacral spinal cord segments of the rat. J. Neuroscience 5(7) : 1729-1739.

SAWADA, M., ICHINOSE, M., & MAENO, T. (1987). Ionic mechanism of the outward current induced by intracellular injection of inositol trisphosphate into Aplysia neurons. J. Neuroscience 7(5) : 1470-1483.

SCHACHER, S., RAYPORT, S.G., & AMBRON, R.T. (1985). Giant Aplysia neuron R2 reliably forms strong chemical connections in vitro. J. Neuroscience 5(11) : 2851-2856.

SCHELLER, R.H., & KIRK, M.D. (1987). Neuropeptides in identified Aplysia neurons : precursor structure, biosynthesis and physiological actions. Trends. Neurolog. Sci. 10 : 46-52.

SCHOT, L.P.C., BOER, H.H., & MONTAGNE-WAJER, C. (1984). Characterisation of multiple immunoreactive neurons in the central nervous system of the pond snail Lymnae stagnalis with different fixatives and antisera absorbed with the homologous and the heterologous antigens. *Histochemistry* 81 : 373-378.

SCHWEITZER, P., MADAMBA, S., & SIGGINS, G.R. (1990). Arachidonic acid metabolites as mediators of somatostatin induced increase of neuronal M-current. *Nature* 346 : 464-467.

SEGAWA, T., & NAKATA, Y. (1991). Substance P receptors in mammalian central nervous system. *Comp. Biochem. Physiol.* 98C(1) : 147-156.

SEWARD, E., HAMMOND, C., & HENDERSON, G. (1991). μ -opioid-receptor mediated inhibition of the N-type calcium channel current. *Proc. R. Soc. Lond. B.* 244 : 129-135.

SHI, R., & BELARDETTI, F. (1991). Serotonin inhibits the peptide FMRFamide response through a cyclic AMP-independent pathway in Aplysia. *J. Neurophysiol.* 66(6) : 1847-1857.

SHI, W-X., & BUNNEY, B.S. (1991). Neurotensin modulates autoreceptor mediated dopamine effects on mid brain dopamine cell activity. *Brain Research* 543 : 315-321.

SIEGELBAUM, S.A., CAMARDS, J.S., & KANDEL, E.R. (1982). Serotonin and cyclic AMP close single K^+ channels in Aplysia sensory neurones. *Nature* 299 : 413-417.

SIVASUBRAMANIAN, P. (1991). FMRFamide-like immunoreactivity in the ventral ganglion of the fly Sarcophaga Bullata: metamorphic changes.

Comp. Biochem. Physiol. 99C(3) : 507-512.

SNYDER, S.H. (1980). Brain peptides as neurotransmitters. Science 209 : 976-983.

SOEJIMA, M., & NOMA, A. (1984). Mode of regulation of the Ach-sensitive K-channel by the muscarinic receptor in rabbit atrial cells. Pflugers Arch. 400 : 424-431.

SONG, Y., & HUANG, L. (1990). Modulation of glycine receptor chloride channels by cAMP-dependent protein kinase in spinal trigeminal neurons. Nature 348 : 242-245.

STANFIELD, P.R., NAKAJIMA, Y. & YAMAGUCHI, K. (1985). Substance P raises neuronal excitability by reducing inward rectification. Nature 315 : 498-501.

STONE, L.S., & MAYERI, E. (1981). Multiple actions of FMRF-amide on identified neurones in the abdominal ganglion of Aplysia. Soc. Neuroscience.Abst. 7 : 636.

STREB, H., IRVINE, R.F., BERRIDGE, M.J., SCHULZ, I. (1983). Release of Ca^{2+} from a non-mitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. Nature 306 : 67-69.

STREB, H., BAYERDOYFFER, E., HAASE, W., IRVINE, R.F., SHULZ, I. (1984). Effect of inositol-1,4,5-trisphosphate on isolated subcellular fractions of rat pancreas. J. Memb. Biol. 81 : 241-253.

SWEATT, J.D., VOLTERRA, A., EDMONDS, B., KARL, K.A., SIEGLEBAUM, S.A., & KANDEL, E.R.. (1989). FMRFamide reverses protein phosphorylation produced by 5-HT and cAMP in Aplysia sensory neurons. Nature 342 : 275-278.

TACHIBANA, K., SCHEUER, P.J., TSUKITANI, Y., KIKUCHI, H., VAN ENGEN, D., CLARDY, J., GOPICHAND, Y., & SCHMITZ, F.J. (1981). Okadaic acid, a cytotoxic polyether from two marine sponges of genus Halichondria. J. Amer. Chem. Soc. 103 : 2469-2471.

TAKAI, A., BIALOJAN, C., TROSCHKA, M., & CASPAR RUEGG, J. (1987). Smooth muscle myosin phosphatase inhibition and force enhancement by black sponge toxin. FEBS Lett. 217(1) : 81-84.

TAYLOR, C.W. (1990). The role of G proteins in transmembrane signalling. Biochem. J. 272 : 1-13.

THOMPSON, S., & RUBEN, P. (1988). Inward rectification in response to FMRFamide in Aplysia neuron L2 : summation with transient K current. J. Neuroscience 8(9) : 3200-3207.

TRIMMER, B.A., KOBIERSKI, L.A., & KRAVITZ, E.A. (1987). Purification and characterization of FMRF-amide like immunoreactive substances from the lobster nervous system: isolation and sequence analysis of two closely related peptides. J. Comp. Neurol. 266 : 16-26.

UEDA, H., NOZAKI, M., & SATOH, M. (1991). Multiple opioid receptors and GTP-binding proteins. Comp. Biochem. Physiol. 98C(1) : 157-169.

UI, M. (1984). Islet activating protein, pertussis toxin: A probe for functions of the inhibiting guanine nucleotide regulatory component of adenylate cyclase. Trends Pharmacol. Sci. 5 : 277-279.

VAN DROP, C., YAMANAKA, G., STEINBERG, F., SEKURA, R.D., MANCLAK, C.R., STIGER, L., & BOURNE, H.R. (1984). ADP-ribosylation of transduction by pertussis toxin blocks the light-stimulated hydrolysis of GTP and GMP in retinal photoreceptors. J. Biol. Chem. 259 : 23-26.

VOLTERRA, A., & SIEGELBAUM, S.A. (1988). Role of two different guanine nucleotide-binding proteins in the antagonistic modulation of the S-type K^+ channel by cAMP and arachidonic acid metabolites in Aplysia sensory neurons. Proc. Natl. Acad. Sci. U.S.A. 85 : 7810-7814.

WALKER, M.W., EWALD, D.A., PERNEY, T.M., & MILLER, R.J. (1988). Neuropeptide Y modulates neurotransmitter release and Ca^{2+} currents in rat sensory neurones. J. Neuroscience 8 : 2438-2446.

WATHER, C., & SCHAFER. (1988). FMRFamide- like immunoreactivity in the metathoracic ganglion of the locust (Schistocerca gregaria). Cell Tissue Res. 253 : 489-491.

WILEY, J.W., GROSS, R.A., LU, Y., & MacDONALD, R.L. (1990). Neuropeptide Y reduces calcium current and inhibits acetylcholine release in nodose neurons via a pertussis-toxin sensitive mechanism. J. Neurophysiol. 63(6) : 1499-1507.

WILLIAMS, J.T., NORTH, R.A., & TOKIMASA, T. (1988). Inward rectification of resting and opiate-activated potassium currents in rat locus neurons. J. Neuroscience 8 : 299-306.

XU, G.P., BEWICK, G.S., & COTTRELL, G.A. (1989). The Helix C3-motoneurone contains ACh and FMRFamide. Comp. Biochem. Physiol. 94C(1) : 321-325.

YAKEL, J.L. (1991). The neuropeptide FMRFamide both inhibits and enhances the Ca^{2+} current in dissociated Helix neurones via independent mechanisms. J. Neurophysiol. 65(6) : 1517-1527.

YANG, H-Y.T., FRATTA, W., MAJANE, E.A., & COSTA, E. (1985). Isolation, sequencing, synthesis, and

pharmacological characterization of two brain
neuropeptides that modulate the action of morphine.
Proc. Natl. Acad. Sci. U.S.A. 82 : 7757-7761.

YATANI, A., CODINA, J., BROWN, A.M., &
BIRNBAUMER, L. (1987a). Direct activation of mammalian
atrial muscarinic potassium channels by GTP regulatory
protein G_k. Science 235 : 207-211.

YATANI, A., CODINA, J., IMOTO, Y., REEVES, J.P.,
BIRNBAUMER, L., & BRAUN, A.M. (1987b). A G protein
directly regulates mammalian cardiac calcium channels.
Science 238 : 1288-1292.